Constitutive and therapeutic benefits of plant resins and a propolis envelope to honey bee, *Apis mellifera* L., immunity and health

A DISSERTATION SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Marla Spivak, Ph.D. Advisor

September, 2015

Acknowledgements

I have so many people to thank that it's difficult to know where to start. Some contributed directly and some indirectly, but all helped me to make this possible.

My path to graduate school may not have been as straight forward as it is for most people, but that is a topic for a whole book. I didn't know I wanted to become a Researcher/Scientist. I liked to work hard, to read and to learn new things. I was working for a beekeeper in Minnesota when I approached Marla (my advisor), and boldly asked her for a job. She not only gave me a job but a year later she hired me as her PhD student, and literally changed my life. I am very honored and grateful to have had Marla Spivak as my advisor and academic mentor. I can't find enough words to thank her for all her support, advices, mentorship, friendship, and of course, patience. Marla believed on my potential when I didn't even know I had any. And for that I will always thank her.

I want to thank the support from members of the Bee Lab. The list may be long, but I would be remiss if I did not mention all of them: Gary Reuter, Michael Goblirsch, Elaine Evans, Morgan Carr-Markell, Katie Lee, Christine Kulhanek, Joel Gardner, Ian Lane, Michael Wilson, Steven Yachik, Michelle Vohs and Brittany Corwin. I would like to express my special thanks to two of them. Gary Reuter, the Bee Lab master beekeeper and "saint" of our field experiments. When I first started in the Bee Lab I was Gary's helper, and I couldn't have had a better beekeeping mentor. Thank you Gary for all your hard work, dedication, uncountable laughs, and for those 25 colonies you let me take from your winter experiment so I could use for my own experiment. You are the BEST! Another special thank you to Dr. Michael Goblirsch, who always had time, or made time, to answer all of my questions (I had many!).

I would also like to extend my sincere gratitude to my committee members, Drs. Adrian Hegeman, Brian Aukema and Timothy Kurtti. I could not have had a better group of faculty. Thank you for all your support, invaluable advice and contributions to my research.

Running helped me in many always during my PhD. It served as a perfect break from my research, a great physical and a different kind of mental challenge. My running shoes were my stress relief tool and always very efficient. Running taught me to always push hard, and having true friends to share those miles with me was a blessing. For that, I want to thank the most amazing runners: Adela Sarahi Chavez and Paulina Bernal, for always keeping me going forward, and fast. And all the members of the Twin Cities Running Club, for always being there for a 6 or 26 mile run, under rain or snow, freezing cold or burning heat!

My family (like a good Brazilian, I have a big one!), a big thank you for the countless words of support. It wasn't easy, but without them it would have been much harder. Thanks to Dayane Lobão, Fernanda Cabrini, Paulo Souza and Angela Kaufmann, my Brazilian family in Minnesota. My sisters, Luciana and Patricia Borba, for all the love and simply being there for me, always! My partner, best friend, and confidant, Fraser Mckee. There is not enough paper in the world for me to properly thank you. Your

unconditional love and encouragement was key to the success of this journey. And last, but never least, my mom and dad. I like to think that I am the most fortunate person in the world because I have an angel in heaven and the most amazing woman in Earth, both, looking after me. My father was the most charismatic person you could possibly know. His smile will forever be in my memory. I hope I made you proud. My mom, I cannot thank my mother enough for everything she has done, and continues to do, for me and my sisters. I know this will sound cliché, but she is my hero. She taught me to be kind, respectful, determined, and most importantly, to be strong and to fight for my dreams. I also hope I made you proud.

Dedication

I dedicate this dissertation to the next generation of the Borba family: Chloe Borba Chasko, Maya Borba Chasko and Ethan Borba Checkver.

“Na vida, não vale tanto o que temos, nem tanto importa o que somos. Vale o que realizamos com aquilo que possuímos e, acima de tudo, importa o que fazemos de nós!” – Chico Chavier

Abstract

Honey bees (*Apis mellifera* L.), like many social insects, have collective behavioral defenses called "social immunity" to help defend and protect the colony against pathogens and parasites. One example of social immunity is the collection of plant resins by honey bees and the placement of the resins on the interior walls of the nest cavity, where it is called a propolis envelope. Propolis is known to have many antimicrobial proprieties against bacteria, fungi, and viruses and has been harvested from bee hives for use in human medicine since antiquity. However the benefit of propolis to honey bees has not been studied until recently. This dissertation research focused on how bees collect and use the antimicrobial properties of plant resins within the hive as a form of social immunity and defense against infectious bacterial and fungal pathogens.

In the first experiment, the benefit of a naturally constructed propolis envelope to individual bee health was assessed by quantifying the gene expression of immune-related genes via real-time PCR, and to colony health by measuring colony strength, pathogen and parasite load of large field colonies. The presence of a propolis envelope within hives of apparently healthy colonies directly affected individual bee health by decreasing the baseline and variability in expression of immune-related genes (such as hymenoptaecin and abaecin) throughout the active foraging season. As the immune system is one of the most costly physiological systems to maintain in animals, a decrease in energetic costs associated with the maintenance of an up-regulated immune system helps bees allocate their energy toward vital tasks (e.g. foraging, rearing brood) and maintain higher storage protein levels in their bodies required for overwintering success. The propolis envelope also benefited colony strength in the spring and increased colony survivorship in one year of the study.

In a second experiment, after colonies were challenged with a highly infectious brood bacterial pathogen, *Paenibacillus larvae*, nurse bee immune system activity and the antimicrobial activity of larval food (fed to young larvae by nurse bees), were both higher when challenged colonies had a propolis envelope compared to when they did not have the envelope. The immune system activity of nurse bees was measured via real-time

PCR, using primers for 3 honey bee antimicrobial peptides (hymenoptaecin, apidaecin and defensin-1). A bacterial growth assay was performed to assess the inhibitory activity of larval food from 1-2 day old larvae against the growth of *P. larvae*. Colonies with a propolis envelope had reduced level of American foulbrood clinical signs (caused by *P. larvae*) two months following challenge, which was likely due to a combination of the effects of propolis on both the collective and individual behavioral responses (larval food bioactivity and individual bee immune response). The results of this experiment reveal a novel therapeutic effect of the propolis envelope and a protective physiological response of nurse bees towards the brood.

The third experiment explored the role of resin collection by honey bees as a general vs. specialized immune defense against the two highly infectious brood pathogens, *Ascosphaera apis* (a fungal pathogen that causes chalkbrood) and *P. larvae* (a bacterial pathogen that causes American foulbrood). The hypothesis was tested that bees self-medicate with resin in response to infection with either pathogen. Results from three years of data suggested that bees significantly increased resin collection, that is, self-medicated the colony with resin, in response to *A. apis* challenge, but not in response to challenge with *P. larvae*. We also tested the hypothesis that bees may shift their selection of resin sources at the colony-level after challenge with the fungal or bacterial pathogen, and, if so, how the antimicrobial activity might differ between the pre- and post-challenge resin plant sources. Resin loads from each bee were analyzed by reverse-phase liquid chromatography mass spectrometry (LC-MS) to identify the plant sources of resin. The inhibitory activity of each resin source against *A. apis and P. larvae* was quantified using dilution assays for each pathogen. After challenge with either pathogen, colonies increased the number of foragers collecting resin from the plants they were already visiting, and not necessarily from the most inhibitory resin. This study sheds light on the complex way in which colony-level behavioral defenses contribute to diminish pathogen infection, and on the role of resins as pharmacological agents in the ecology and evolution of plant-animal interactions. Further research will be necessary to investigate whether bees self-medicate with resin based on resin quality or available quantity.

In all, these studies demonstrate the significance of the propolis envelope as a crucial component of the nest architecture in honey bee colonies. The collection and deposition of resins into the nest architecture impacts individual immunity, colony health, and induces honey bees antimicrobial defenses. These results emphasize the importance of resin to bees and show that plants are not only a source of food but can also be "pharmacies."

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Introduction

Honey bees, *Apis mellifera* L., are the world's most important pollinator of many of our food crops, and essential to the productivity of agro- and natural ecosystems (Gallai et al., 2009). The pollinating capacity and overall health of honey bees in the U.S. have come under severe threat due to interacting effects of pathogens, parasites and poor nutrition (Cox-Foster et al., 2007; Johnson et al., 2009; Runckel et al., 2011). Beekeepers are losing an average of 30% of their colonies annually (vanEngelsdorp et al., 2011) due to these multiple effects. Beekeepers have been recovering the losses by splitting surviving colonies or by purchasing new colonies from national bee breeders and distributors, however this process is not economically or biologically sustainable. It is critical to improve the health of surviving bee colonies, which are now serving as the genetic reservoir for all bees in the U.S. One way to improve bee health is to understand and promote their natural defenses against pathogens and parasites. My thesis is on an important but highly understudied natural defense of honey bees: their use of plant resins.

The main goal of my dissertation was to explore how resin collection and deposition within the nest cavity as "propolis" impact individual bee immunity and overall colony health, strength and survivorship in the presence or absence of pathogen stress. A long term, and personal, goal for this dissertation was to study ways for beekeepers to encourage honey bee colonies to deposit a propolis envelope within standard beekeeping equipment, to then modify the equipment currently used for beekeepers and beekeeping practices nationwide.

Like many social insects, honey bees have collective behavioral defense mechanisms that assist the immune system of individual bees (Evans et al., 2006). These behavioral defenses are called "social immunity" (Cremer et al., 2007). One example of social immunity is honey bee hygienic behavior, the genetic ability of bees to remove diseased and parasitized brood from the nest (reviewed in Wilson-Rich et al., 2008; Evans and Spivak, 2010). Another form of social immunity is the collection of plant

resins by honey bees and the placement of the resins in the nest as a form of cement, called propolis by beekeepers (reviewed in (Simone-Finstrom and Spivak, 2010).

Some plants secrete highly aromatic resins that have a number of antimicrobial properties, which serve to protect the plant against predators and pathogenic microorganisms (Langenheim, 2003). In a natural nest habitat (tree cavities), bees collect plant resins and deposit them on the entire inner surface of the nest wall, constructing a propolis envelope (Seeley and Morse, 1976). Previous research in the Spivak lab tested the benefits of propolis to the social immunity of honey bee colonies by experimentally coating the inside of boxes with a propolis extract solution (ethanolic solution of propolis) to simulate a propolis envelope surrounding the colony. After just seven days exposure to the propolis enriched nest environment, bees' immune-related gene transcription and bacterial load was significantly lower compared to bees in boxes not enriched with the propolis-extract (Simone et al., 2009). These results suggested that the propolis reduced the level of immune-elicitors in the nest, so that the bees were able to expend less energy on costly immune system activation (Simone et al., 2009). Ironically, beekeepers, particularly in the U.S., have selected against colonies that collect large amounts of propolis (Fearnley, 2001) because its stickiness makes opening and managing colonies in commercial beekeeping equipment difficult. Importantly, honey bees are not able to construct a natural propolis envelope within standard beekeeping equipment because the inner walls of the pine boxes are smooth and do not elicit resin collection. Instead the bees deposit propolis only in dispersed cracks and crevices and not as a continuous envelope.

In the same way that resin secretion by a plant may be constitutively produced and/or actively induced to assist in plant defense (Langenheim, 2003), honey bees may collect resins and deposit propolis constitutively and/or actively to benefit the health of the colony. Other research in the Spivak lab, demonstrated that colonies with a propolis envelope had reduced levels of a fungal pathogen, *Ascophaera apis*, that causes chalkbrood infection in bee larvae and pupae (Simone-Finstrom and Spivak, 2012). Additionally, colonies increased resin foraging at the colony level after exposure to *A.*

apis, revealing that that bees medicate the colony with propolis in response to this fungal infection (Simone-Finstrom and Spivak, 2012).

In a collaborative effort among Dr. Spivak in the Department of Entomology, and Drs. J. Cohen, A. Hegeman and G. Gardner of the Department of Horticultural Science, Wilson et al. (2013) quantified the bioactivity of resins from different botanical sources against *P. larvae* growth. That study revealed a significant difference among resins from 14 tree species collected on the St. Paul campus of the University of Minnesota in their ability to inhibit the growth of this bacterium. Likewise, propolis samples from different locations across the U.S. and in Brazil were found to have very different inhibitory activities against the growth of *P. larvae* and *A. apis in vitro* (Bastos et al., 2008; Wilson et al., 2015). The collection of resins by bees from diverse botanical sources, each with their own complex mixtures of antimicrobial compounds, likely increases the potential of bees to benefit from a broad spectrum of activities against microbes. However, it is not known if honey bees can discriminate among resins and deliberately collect resin from specific trees, a behavior displayed by other bees. Stingless bees use olfactory cues to find resin sources and select resin sources on the basis of several volatile compounds (mono- and sesquiterpenes; Leonhardt et al., 2010).

My dissertation had three main objectives. I tested:

1. The seasonal benefits of a propolis envelope to colony health and individual honey bee immunity.
2. The therapeutic role the propolis envelope plays in bees' natural defense against the bacterial brood pathogen, *Paenibacillus larvae*.
3. How honey bees select and use plant resins as a form of self-medication in response to *Paenibacillus larvae* or *Ascosphaera apis* challenge.

In Chapter 1, I provide a summary of all my findings (chapter 2, 3 and 4). I was invited to write this chapter for a book entitled "Beekeeping Science for Beekeepers" to be published by Springer-Verlag in 2016. The editors of the book had specific writing requirements for this chapter: it must be written in "non-jargon oriented language for non-scientists beekeepers and bee-lovers." Beekeepers spend a significant amount of time

reading about bee related discoveries. In some cases, because of highly technical language and complex results, they miss the main points and/or misinterpret the data. I have always been interested in both basic and applied research, and feel it is very important to transfer sound scientific knowledge to promote honey bee health and to develop and inform beekeeping management decisions with the goal to increase the sustainability of beekeeping operations. Thus, I am honored to contribute to this book.

I am including Chapter 1 in lieu of a more formal literature review chapter normally found in a dissertation. Simone-Finstrom and Spivak (2010) wrote a thorough review of the natural history and resin use by honey bees. Because this is an understudied research area, relatively few contributions have been made to the scientific literature since the previous review (e.g. Bilikova et al., 2013; Mao et al., 2013; Nicodemo et al., 2013; Nicodemo et al., 2014; Wilson et al., 2013; Popova et al., 2014; Wilson et al., 2015). Therefore, a small literature review can be found in my introductions to Chapters 2, 3 and 4.

In Chapter 2, my aim was to examine the relative immune and health benefits of the natural propolis envelope from the scale of the individual bee to the level of the entire colony. Previous work by Simone et al. (2009) reported the benefits of propolis to bee health, after seven days of exposure, by coating the inside of small colonies with a propolis extract paint (solution of propolis in 70% ethanol). As organic solvent extracts of propolis may not contain all active compounds, and is not how bees are exposed to propolis naturally, I allowed the bees to construct their own propolis envelope, in standard size bee colonies, and investigated the benefits of the propolis envelope over the course of a year.

I developed and tested a method to stimulate bees to construct a natural propolis envelope within the nest of commercial hives. After bees had created the propolis envelope, I examined the effect of a naturally constructed propolis-envelope within standard beekeeping equipment on the strength, pathogen and parasite load of large field colonies, and immune system activity, virus and storage protein level of individual bees

over the course of a year. This chapter has been submitted to the Journal of Experimental Biology and is under review.

In Chapter 3, using another set of colonies, I studied if a natural propolis envelope in a honey bee hive helps promote a therapeutic defense at the individual and colony level after challenge with *Paenibacillus larvae*. I tested the effects of the propolis envelope as a natural defense against *P. larvae* on the antimicrobial activity of larval food, the expression of antimicrobial peptides in 7-d old nurse bees, and the overall reduction of clinical signs of AFB by the colony. I had the idea of investigating the inhibitory activity of larval food to *P. larvae* growth after reading about social immunization behaviors, in ants, to fight bacterial and fungal pathogens (Hamilton et al., 2011; Konrad et al., 2012). I found two very novel findings: 1) bees in colonies with a propolis envelope increased immune system activity after challenge, revealing that the immune system is not suppressed in the presence of a propolis envelope, and 2) the bioactivity of larval food was significantly higher in challenged colonies with a propolis envelope compared to in challenged colonies without the propolis envelope. This study emphasizes the critical importance of the propolis envelope to honey bee health and demonstrates its therapeutic role to both larvae and adults.

In Chapter 4, I studied how honey bees, select and exploit the antimicrobial properties of resin for the health of the colony. I challenged honey bee colonies with a bacterial pathogen (*Paenibacillus larvae*), and another set of colonies with a fungal pathogen *Ascosphaera apis*) and investigated if bees medicate the colony ("self-medication"; Clayton and Wolfe, 1993) with resin in response to either pathogen compared to unchallenged colonies. Based on the hypothesis that bees are able to discriminate among resins and display adaptive plasticity in resin botanical source collection, I also explored if bees change their foraging preference to sources with greater biological activity after challenge with a bacterial (*P. larvae*) or fungal (*A. apis*) pathogen. This study was a collaborative work between the Spivak lab and two other labs in the Horticulture Department (University of Minnesota, Twin Cities campus), Drs. Hegeman, Cohen and Wilson.

Our results strongly indicate that the propolis envelope serves as an external antimicrobial layer around the colony, providing fundamental constitutive benefits to adult bees' immunity, greater colony fitness in early spring. When colonies are challenged with a bacterial pathogen, the propolis envelope confers therapeutic protection to the brood from *P. larvae* infection and supports nurse bees' ability to induce a strong and effective immune response after infection, resulting in a lower infection load after two months following bacterial challenge. Finally, the last section of this dissertation lends more support to the idea that honey bee self-medicate with resin foragers after *A. apis* challenge, opening doors to new research on how or if bees select resin sources, and how recruitment by nestmates affects resin collection.

There is still much research to be done to fully understand how propolis can be leveraged to benefit beekeepers. Future researches on the effect of the propolis envelope to other honey bee pathogens, to the honey bee microbiota, and to overwintering success are needed, and are areas I am interested in pursuing. More investigations on bees' natural defense behavioral mechanisms to fight pathogen and parasite infection have the potential to greatly improve bee health and positively impact the beekeeping industry.

Chapter 1.
Summary of research findings

1.1 Benefits of propolis to colony health

It is common knowledge that honey bees forage for pollen, nectar and water. What is not well appreciated is that honey bees also forage for plant resins, but not for nutritional reasons. Resin is a sticky exudate secreted prophylactically by plants to protect young leaf buds or the entire plant from disease, UV light, and herbivore attack (Langenheim, 2003). Resins are composed primarily of antimicrobial compounds (e.g. monoterpenes and flavonoids) that play a major role in the defense and survival of the plant (Langenheim, 2003). Our research has found that these antimicrobial resins also play a major role in the immune defense and health of honey bee colonies.

Honey bees collect resin mainly from buds and leaves of various tree species, but they also collect resins from droplets appearing on the trunks or limbs of trees (Alfonsus, 1933), from the surfaces of some fruits (e.g., *Macarangatanarius*; Kumazawa et al., 2003), or as a reward for pollination of some flowers [e.g., *Clusia* (Clusiaceae) and *Dalechampia* (Euphorbiaceae)] (Armbruster, 1984). Bees can extract resin by fragmenting leaves with their mandibles (mouthparts) or collect it directly from the plant surface (Meyer, 1956; Teixeira et al., 2005). Bees collect resins to varying degrees; some honey bee species and races use resins extensively; for example African-derived subspecies *Apis mellifera scutellata*, and European-derived subspecies *A. mellifera caucasica*. At least one species of honey bees, *Apis cerana*, is reported to collect no resin (Butler, 1949; Page and Fondrk, 1995). In colonies that do collect resin, the number of resin foragers depend on the needs of the colony (as discussed later in this chapter), but generally they are less than 1% of the total forager work force at any point in time. Resin collection is a very difficult and time consuming task to perform. After chewing pieces of resin from the plant, bees must transfer the sticky secretion from their mandibles to their hind legs before returning to the hive. Because of the sticky characteristics of resin, once

back in the hive, resin foragers need the assistance of other bees to remove the resin load from their legs, which may take up to 30 minutes (Nakamura and Seeley, 2006). The bees will then carry the resin in their mandibles to the site in the hive where the resin will be deposited. Once deposited in the nest, the resin, sometimes mixed with beeswax, is called propolis.

Honey bees naturally nest in tree cavities where they coat the entire inner surface of the nest cavity surrounding the combs with a propolis envelope (Seeley and Morse, 1976). It was suggested by Seeley and Morse (1976) that the propolis envelope had various functions, including serving as an impermeable barrier to tree sap and environmental moisture, a solid surface for comb attachment, a physical barrier to outside invaders by sealing the holes and cracks of the nest cavity, and finally, an antimicrobial layer against natural occurring fungi and bacteria in the tree cavity. When nesting in a hollow tree cavity, honey bees prepare the new nest site by removing the soft, rotten wood from the nest walls and depositing propolis in the cracks and top surface to make it solid and smooth (Seeley and Morse, 1976). Beekeepers, particularly in the U.S., have selected against colonies that collect large amounts of propolis (Fearnley, 2001) because its stickiness makes opening and managing colonies in standard beekeeping equipment difficult. Importantly, honey bees do not construct a propolis envelope within standard beekeeping equipment because the inner walls of the wooden boxes are already solid and smooth, which apparently does not stimulate bees to deposit propolis on them. Instead, bees deposit propolis in dispersed cracks and crevices in manmade bee boxes, and not as a continuous envelope as they do within a tree cavity (reviewed in Simone-Finstrom and Spivak, 2010).

Honey bees are very resilient insects; they have thrived in this world for 6-8 million years (Engel, 1999), relying only on their own natural defense mechanisms to survive. Although propolis has been used as a traditional and natural human medicine since biblical times (Simone-Finstrom and Spivak, 2010), the benefits of propolis for honey bee health were not appreciated until we began research on this topic in the last decade. Our research has shown that the presence of a propolis envelope enshrouding the nest

area is a fundamental component of honey bee colony health. The propolis envelope functions as an antimicrobial, or disinfectant layer around the nest, and thus as an external layer of the colony immune system. This chapter will summarize current research questions we have explored in the past few years, since the previous review (Simone-Finstrom and Spivak, 2010), including: 1) the seasonal benefits of a propolis envelope to colony health and individual honey bee immunity; 2) the therapeutic role the propolis envelope plays in bees' natural defense against brood diseases; and 3) how honey bees select and use plant resins as a form of self-medication.

1.2 Seasonal benefits of propolis to bee immunity and colony health under natural field conditions

A honey bee colony can be considered a superorganism, a group of related individuals living together in a nest with the ability to perform collective foraging, thermoregulatory and defensive behaviors. When collective behavioral mechanisms are used to defend the colony against parasites and pathogens, they are called mechanisms of social immunity (Cremer et al., 2007). Examples of social immunity in honey bees include hygienic behavior (the ability of adult bees to detect and quickly remove diseased and mite infested brood from the nest, limiting pathogen and parasite transmission; reviewed in Evans and Spivak, 2010), grooming (removal of the parasitic *Varroa* mite from a nestmate's body; Boecking and Spivak, 1999) and foraging for resins to form a propolis envelope inside the nest (Simone et al., 2009; Simone-Finstrom and Spivak, 2012).

The benefits of the propolis envelope to honey bee health were first investigated in our lab at the University of Minnesota by coating the inside of small managed hives with a propolis extract (solution of 13% propolis in 70% ethanol) with a paintbrush, and allowing bees to be exposed to this propolis-enriched environment for 7 days (Simone et al., 2009). After one week, 7-day old bees had lower immune system activation and lower

bacterial loads in and on their bodies compared to same-age bees in hives without the propolis-extract coating (Simone et al., 2009). These initial findings told us that bees in hives with the propolis envelope did not have to expend as much energy turning on (activating) their immune system to fight off microbes, presumably because there were fewer microbes in the nest. When the immune system of bees, or any animal, is activated it comes with a physiological cost such as reduced survival (Moret and Schmid-Hempel, 2000). In fact, the immune system is the most costly physiological system to maintain (Evans and Pettis, 2005; Schmid-Hempel, 2005). When the immune system does not need to be highly activated, as when there is a propolis envelope in the nest cavity, bees are able to allocate their energy to perform vital tasks (e.g. foraging, rearing brood) and to store protein in their bodies.

Following up on Simone-Finstrom's Ph.D. research on the short-term benefits of the propolis-extract coating inside the bee hive, we were curious to know the long-term benefits of a propolis envelope that was naturally deposited by the bees. Recent research from Brazil showed that Africanized bee colonies that collect high amount of propolis, had greater brood viability, longer worker lifespan, higher honey production, more rapid hygienic behavior and larger pollen stores, compared to colonies that collect low amount of propolis (Nicodemo et al., 2013; Nicodemo et al., 2014). We wondered if our European-derived honey bees in the U.S. could also receive the same long-term benefits from the antimicrobial compounds in propolis. As most European-derived stocks of bees in the U.S. do not collect much propolis, we encouraged colonies to build a natural propolis envelope. We cut and stapled commercially available propolis traps to the four inner walls of each hive box in 12 colonies (propolis envelope treatment group), and the bees readily filled the 3mm gaps in the traps with resin they collected from the field (Figure 1.1). No propolis traps were provided to another set of 12 colonies, and the bees deposited propolis in the cracks and crevices within the box only where they could (control group). This experiment was conducted on a first set of colonies from April 2012 to May 2013 and was repeated on a new set of colonies from April 2013 to May 2014. Each year the colonies were started from package bees on unused equipment and combs.

During the active foraging season (from July to September) and the following May, of both years, we quantified: 1) the adult bee population in each colony by counting the number of frames covered with bees in each box (following Nasr et al., 1990); 2) the total amount of worker brood by placing a 2.6 cm^2 grid over each frame and counting the number of squares filled with sealed or unsealed brood (following Nasr et al., 1990); and 3) levels of *Varroa* mites and *Nosema* by collecting samples of 300 adult bees from the brood area to quantify the levels in the laboratory (following Lee et al., 2010; Spivak and Reuter, 2001a).

We also measured the effects of propolis on individual bee health by quantifying the expression of specific immune genes. The honey bee immune system is similar to ours, with one major exception: honey bees do not produce antibodies that "remember" specific antigens (e.g., pathogens). Instead, bees rely on more basic responses, such as the production of antimicrobial peptides to break down pathogens (humoral immunity), or the engulfing and encapsulation of pathogens by molecules in bees' blood cells (cellular immunity). The starting point of humoral immune system activation, called gene transcription, can be measured using a common (but somewhat expensive) laboratory technique called real-time, quantitative PCR (Polymerase Chain Reaction). PCR amplifies a specific sequence of genes into millions of copies, which are then quantified by the real-time PCR machine; the more copies, the higher the abundance of the gene transcription product. A specific sequence of genes is targeted using "primers", which are small segments of DNA that consist of a sequence of nucleotides that are complementary to a piece of the gene of interest for replication, in our case, genes responsible for immune system activation.

We also used real-time PCR to quantify the levels of three different viruses in the bees (DWV – Deformed Wing Virus; IAPV – Israeli Acute Paralysis Virus; BQCV – Black Queen Cell Virus) using primers specific to those viruses. Finally, we measured the level of vitellogenin (Vg), an important protein in bees' hemolymph as it is an indicator of well-nourished bee and contributes important priming function to the

immune system of bees (Amdam et al., 2003; Amdam et al., 2004; Engels et al., 1990; Salmela et al., 2015).

To measure immune system activation, it is best to collect bees of the same age, preferably young nurse bees, as the immune systems of older foragers become highly variable in expression levels. To collect young bees, we paint-marked newly emerged bees with a dot of enamel paint on the thorax just after they crawled out of their cells. We returned to the hive six days later to collect the painted 7-day old bees from each of the experimental colonies. We measured the immune system activation in bees in the summer, fall and the following spring. Honey bees in Minnesota do not forage for resin (or for any resources) during our long cold temperature season, from October to April, and we were curious to know if the effect of propolis collected the previous summer and fall would retain its benefit by the following spring, in early May, before the trees were producing new resins. We used 25 painted bees per colony at each time point to measure the immune system activity, virus and blood protein levels.

Our results clearly supported and extended our earlier work (Simone et al., 2009): the naturally constructed propolis envelope served to lower immune gene expression in bees over the summer and fall months, resulting in a much "quieter" immune system compared to bees in control colonies. A decrease in energetic costs associated with the maintenance of an efficient immune system helps bees to allocate their energy to perform vital tasks (e.g. foraging, rearing brood) and to maintain higher storage protein levels (e.g. Vg) required for overwintering success. In addition to the direct effect on bees' immune system, we found that bees in colonies with a propolis envelope had a less variable (more uniform) immune gene expression over the active foraging season, potentially representing a healthier population (Dawkins et al., 2013).

Although we studied the expression of six immune related genes, we show here the results for only two of the immune genes from 7-d old bees in July, September and the following May of the first year of this experiment, as an example of the data set (Figure 1.2). The expression level of the two immune genes, hymenoptaecin and abaecin, were significantly lower in bees from propolis envelope colonies (Figure 1.2, as noted by the

lower vertical bars), compared to bees in colonies without the propolis envelope, in July and September. The second year had very similar results, particularly over the summer months.

In spring of both years, before the bees were actively collecting resin again, there were no significant differences in gene expression levels for most immune genes between bees from the two treatment groups. Using a test described later in this chapter (see 1.4), we found that the propolis within the nest in late April had lost much of its antimicrobial activity from the previous fall, which means that in the spring of the following year, the direct effect of the propolis on the immune system of honey bees was minimal. The loss of biological activity of the propolis from October to April is probably due to the lack of new resins being brought in over the winter. In Minnesota, bees start collecting resin again later in May, when environmental temperatures for tree growth are favorable.

Measures of the blood storage protein, vitellogenin, which is an indicator of the nutritional health of bees (Engels et al. 1990; Amdam et al., 2003; Amdam et al., 2004), were significantly higher in spring of both years (May 2013 and May 2014) in bees from colonies with a propolis envelope compared to bees from control colonies (Figure 1.3). This high Vg level in bees from propolis envelope colonies in the spring of both years suggest that these bees had more protein storage compared to bees in control colonies and, therefore, were able to rear more brood than control colonies (Bitondi and Simoes, 1996; Mattila and Otis, 2006). We found that colonies with a propolis envelope had significantly larger brood areas in May 2013 compared to controls, but this difference was not significantly different in May 2014 (Figure 1.4). Additionally, more colonies with a natural propolis envelope were alive at the end of the experiment in 2013, but not in 2014.

The levels of pathogens (viruses, parasitic mites and *Nosema*) were very low and did not differ between the colonies in the two treatment groups. The low levels of pathogens were because all colonies began as “packages” and normally mite and pathogen levels do not rise to high levels in new colonies the first year in our area (personal observation). In contrast to previous findings (Simone et al. 2009), the levels of

bacteria on bees' bodies (measured by the transcript abundance of the eubacterial rRNA 16S gene) were not lower in bees from our colonies with a propolis envelope. Nonetheless, both studies found that bees in propolis-rich colonies had quieter immune systems compared to bees in propolis-poor (control) colonies. The lack of difference of bacterial levels between our treatment groups, but a significant decrease in immune gene expression in bees from propolis envelope colonies, suggests that propolis had a direct effect on the bees' immune system. These results also classify the benefits of the propolis envelope to bees as a constitutive benefit, which means it is always present and not as a result of a pathogen infection (induced).

In sum, colonies that are allowed to construct a natural propolis envelope on the inside of the hive boxes benefitted in ways that improve bee health and possibly colony strength and survivorship. The propolis envelope creates an antimicrobial layer around the bees that, remarkably, serves as an environmentally derived component of the bee's immune system. The propolis helps the bees' immune system either by reducing the microbe load in the nest cavity, as suggested by Simone et al., (2009), or by having a direct and beneficial effect on bees' immune system.

A Human Analogy. To fully understand the function and benefits of the propolis envelope to bees, it is helpful to draw an analogy between a honey bee nest and human homes. Mold and fungi are often found in our houses, especially during spring and summer when humidity is higher. The presence of these microorganisms in the air may not always cause a health problem, but some people's immune systems are easily affected by these microorganisms and the inhalation of molds and fungi can lead to immune activation in more sensitive people. The propolis envelope to bees would be the same as coating the walls of our homes with an antimicrobial material. In that case, the antimicrobial material would: 1) effectively decrease the levels of microorganisms growing on the walls of the house and indirectly preventing our immune system from activating an immune response and 2) directly decrease the expression of immune genes. Mounting a strong immune response comes with a cost, so the lower immune system activation is beneficial. The immune system needs to use energy to fight off pathogens

and when it is always activated, the individual, whether bee or human, is left with less body resources and greater immune stress, which may affect overall health and ability to fight off secondary or subsequent infections.

1.3 The therapeutic role of the propolis envelope for bees' natural defense against brood diseases

In addition to the every day (constitutive) benefits of the propolis envelope to the bees' immune system (as described in 1.2), the antimicrobial properties of propolis can promote a therapeutic defense against pathogens. A recent study found that when honey bee colonies that contained a propolis-extract coating (experimentally applied envelope) were challenged with chalkbrood, a brood pathogen caused by the fungus *Ascosphaera apis*, they had less chalkbrood infected brood (average of 14.7 ± 7.5 chalkbrood infected larvae per colony) compared to colonies with no propolis-extract coating (108.2 ± 49.0 chalkbrood infected larvae per colony; Simone-Finstrom and Spivak, 2012). We do not understand the mode of action by which the propolis decreases clinical signs of chalkbrood in honey bee colonies, but these initial findings were intriguing and led us to test the effect of a natural propolis envelope on a different disease, American foulbrood.

American foulbrood (AFB) disease is caused by the bacterial pathogen, *Paenibacillus larvae*. AFB is highly infectious to honey bees and can rapidly spread among colonies via drifting (when a forager enters a colony that is not their own) and robbing of contaminated nectar. Young honey bee larvae (1-2 days old) are highly susceptible to this pathogen, while old larvae and adults are considered resistant. A potential reason for this susceptibility is thought to be because young larvae may have "weaker" immune defenses compared to older brood and adults (lower bee "blood" cell counts and cellular defense mechanisms; Chan et al., 2009; Wilson-Rich et al., 2008).

Previous studies have demonstrated four different mechanisms of colony resistance to AFB: 1) removal of *P. larvae* spores from contaminated honey by action of the honey stopper (Sturtevant and Revell, 1953); 2) detection and rapid removal of AFB-infected

brood by adult bees before the pathogen becomes infectious (hygienic behavior; Spivak and Reuter, 2001); 3) genetic ability of larvae to resist AFB infection (Evans, 2004; Rothenbuhler and Thompson, 1956), and 4) ability of nurse bees to secrete antimicrobial compounds into larval food, which can protect the larvae somewhat from *P. larvae* infection (Rose and Briggs, 1969; Thompson and Rothenbuhler, 1957). Additionally, numerous studies have demonstrated that propolis has antimicrobial properties that inhibit the growth of *P. larvae* (Bastos et al., 2008; Bilikova et al., 2013; Wilson et al., 2013; Wilson et al., 2015). Therefore, we explored whether a natural propolis envelope could promote a fifth mechanism of defense against AFB.

We posed three questions in our experiment. 1) After colonies were challenged with the bacterium that causes AFB, would the level of antimicrobial peptides (immune system activation) be higher in nurse-age bees in colonies with a propolis envelope compared to nurse bees in colonies without the envelope? 2) Would the antimicrobial activity of larval food supplied by nurse bees to young larvae be higher in colonies with a propolis envelope? And, 3) Would there be less AFB-infected brood in colonies with a propolis envelope?

In the summer of 2013, we stimulated ten colonies to construct a propolis envelope by stapling propolis traps to the inner walls of standard beekeeping boxes (as explained in 1.2). We then experimentally challenged five of the 10 colonies with *P. larvae* by spraying a sugar solution with a known concentration of *P. larvae* spores on each comb within the colony (propolis + *P. larvae* treatment). The other five colonies with a propolis envelope were left unchallenged (propolis + no *P. larvae* treatment). Another set of ten colonies was not provided with a propolis envelope and the bees deposited propolis in the cracks and crevices within the box where they could. Similarly, five of the ten colonies without a propolis envelope were challenged with *P. larvae* (no propolis + *P. larvae* treatment) and the other five were left unchallenged (no propolis + no *P. larvae* treatment).

We collected samples of 7-day old bees to test the expression levels of immune genes (as explained in 1.2), and samples of larval food to test its antimicrobial activity

twice during the experiment, once before and once after the challenged colonies showed clinical signs of AFB (August 9th and September 12th, respectively). The number of larvae with clinical signs of AFB (sunken wax capping and uncapped cells containing discolored, ropy brood) was quantified approximately every 15 days after the appearance of the first clinical sign (August 30th, September 16th and October 1st).

The antimicrobial activity of larval food was measured in liquid culture. Most bacteria, such as *P. larvae*, can be grown under controlled laboratory conditions, in tubes containing a liquid with the required nutrients for bacterial growth (called broth). Bacterial growth in liquid culture is characterized by the increased turbidity of the culture, and the optical density (OD) of the liquid culture can be measured using a spectrophotometer. This machine produces a light of a pre-selected wavelength in one end of the chamber that houses the sample, and records the intensity of light detected at the other end of the chamber after it passes through the sample. Samples with a greater concentration have a greater optical density and will absorb more light, reducing the intensity of light that reaches the receptor. Therefore, the intensity of the light detected by the receptor decreases as the sample concentration, and optical density, increases. Our antimicrobial activity assay consisted of allowing a known concentration of *P. larvae* in broth brain/heart infusion (BHI) to grow in larval food for 6 hours at 37°C and subsequently evaluating the bacterial growth by measuring the optical density (OD at time 0h subtracted from time 6h). We compared bacterial growth in cultures with brood food relative to cultures without brood food (controls).

Immune gene expression analysis of nurse age bees collected after the *P. larvae* challenge showed that bees from colonies with a propolis envelope had a stronger immune response compared to bees in colonies without a propolis envelope, as indicated by significantly higher gene expression levels of two antimicrobial peptides (hymenoptaecin and apidaecin). These results indicate that nurse bees from propolis envelope colonies have the ability to synthesize higher levels of antimicrobial compounds and potentially decrease colony-level AFB infection more rapidly and efficiently compared to bees without a propolis envelope. Importantly, these findings demonstrate

that bees in colonies with a propolis envelope are able to mount a strong immune response after they are challenged. Thus, the lower immune system activation ("quieter" immune system) of bees in apparently healthy colonies with a propolis envelope (see 1.2) is *not* due to immune suppression (ie., the inability to mount an immune response), because after challenge these bees are able to quickly activate their immune responses.

Nurse bees perform the behavioral task of feeding the brood by regurgitating larval food into the cells and therefore, are in constant direct contact with the susceptible larval stage to AFB. We found that when the challenged colonies had a propolis envelope, the bioactivity of the larval food was significantly higher compared to the larval food in unchallenged colonies without a propolis envelope (Figure 1.5). The higher antimicrobial activity of larval food in challenged colonies with a propolis envelope reveals a therapeutic effect of the propolis envelope. One hypothesis to explain these findings is that volatile compounds from the propolis envelope contribute directly to the bioactivity of larval food against bee pathogens. Although the propolis envelope may not come into direct contact with larval food, volatile compounds present in propolis can diffuse through the hive, and may contribute to the complex way in which bees fight infections. Another hypothesis is that nurse bees produce more antimicrobial peptides after they are challenged, and incorporate these antimicrobial peptides (Bilikova et al., 2001) into larval food fed to 1-2 day old larvae to increase young larvae immune defense mechanism to fight *P. larvae* infection. Either way, our results confirm the existence of a natural defense mechanism in honey bees against AFB by feeding larvae food with a higher antimicrobial activity (Rose and Briggs, 1969; Thompson and Rothenbuhler, 1957). Importantly, this mechanism of defense against AFB was only observed when colonies had a propolis envelope.

Clinical signs of AFB can be identified by the presence of sunken wax cappings and uncapped cells containing discolored, ropy brood. As a measure of level of AFB infection, we counted, in each comb, the number of cells containing signs of AFB (Spivak and Reuter, 2001b). An overall infection level on each inspection date (August, September and October) was obtained by calculating the mean (± standard error; Table

1.1) of the total number of AFB-infected cells for each colony. Our results indicate that the presence of a propolis envelope inside a colony reduced the number of larvae with clinical signs of AFB over time, but did not eliminate the disease completely. Simone-Finstrom and Spivak (2012) study on the effect of a propolis-rich environment to the infection level of chalkbrood disease, reported that colonies with a propolis-extract coat inside the nest had a level of infection 86% lower (14.7 ± 7.5 cells compared to 108.2 ± 49.0) than observed in colonies without the propolis extract. Similarly, our findings show that colonies with a propolis envelope had 77% less cells infected with AFB in October compared to colonies without a propolis envelope (Table 1).

In sum, we demonstrate a new mechanism of how a bee colony can fight AFB disease: the presence of a propolis envelope, which increases the individual and collective immune responses of bees through the production of antimicrobial peptides in individual nurse bees, and the increased bioactivity of larval food fed collectively by nurse bees. We suggest that the reduced level of AFB clinical signs in early October in colonies with a propolis envelope compared to colonies without a propolis envelope is a result the combined effects of the natural propolis envelope. The propolis envelope served as an external antimicrobial layer around the colony, protecting the brood from *P. larvae* infection and supporting bees' ability to induce a strong and effective immune response with the result of a lower infection load after two months following the challenge.

1.4 Do bees self-medicate?

Self-medication is defined as the "defense against pathogens and parasites by one species using substances produced by another species" (Clayton and Wolfe, 1993). If bees can truly self-medicate, an individual (or colony) should perform a behavior, such as resin collection, at higher rates when parasitized and at lower rates when healthy. Simone-Finstrom and Spivak (2012) found that honey bee colonies increase resin

foraging after exposure to chalkbrood, revealing that bees medicate the colony with resin in response to this particular fungal infection. To extend our knowledge of how honey bees exploit resin to fight pathogen infection, we investigated whether bees also self-medicate in response to a bacterial infection, American foulbrood (AFB).

We used small colonies, equalized in population size and food resources, and monitored the resin foraging activity when the colony was healthy and after experimentally challenging them with either *Paenibacillus larvae*, the causative agent of AFB, or *Ascosphaera apis*, the causative agent of chalkbrood (CB). The number of resin foragers was assessed before challenge by closing the colony entrance for 15 minutes for 12 observation periods (spread over two weeks), and recording the number of returning foragers with a resin load on the hind legs. After 12 observations, one group of colonies was challenged with a *P. larvae* spore solution (as mentioned in 1.3), and another group was provided with a pollen patty containing *A. apis* spores. A third group of colonies served as controls (unchallenged colonies). Resin foragers were again counted over another set of 12 observations periods spanning two weeks. The increase in resin foraging after colony inoculation was measured as the difference between the total number of resin foragers after challenge per colony minus the total number before challenge per colony, and this difference was compared among treatment groups (control, AFB- and CB-challenged colonies). This study was repeated over three years from 2012 to 2014 using new sets of colonies each year.

Our results show that colonies challenged with *P. larvae* had a numerical increase in resin foraging, but not statistically different to unchallenged colonies. Therefore, we are not able to say that bees self-medicate by collecting more resin after *P. larvae* challenge. A numerical increase means there was a trend, but the increase could have happened simply by chance rather than due to the challenge. A statistically significant increase means that the increase in resin collection was due to the pathogen challenged. Our findings do strongly support previous results that bees self-medicate with resin in response to fungal infection from *A. apis* (Simone-Finstrom and Spivak, 2012), as found that bees significantly increase resin foraging after challenge with *A. apis* (Figure 1.6).

Do bees self-medicate with specific plant sources of resin? When we are sick, we can go to the pharmacy and self-medicate by buying an over-the-counter drug that treats the infection we are experiencing (e.g. bacterial or fungal infection). Honey bees self-medicate in a similar way by collecting antimicrobial resins ("drugs") from plants ("pharmacy"), but we do not know if bees choose specific resins that are most able to treat the infection the colony might have.

Chemical composition of resins varies qualitatively and quantitatively within and among plants (Witham, 1983). Wilson et al. (2013) study of the bioactivity of resins from different botanical sources against *P. larvae* growth revealed a significant difference among resins from 14 tree species on their ability to inhibit the growth of this bacterium. Likewise, previous research has found that propolis samples from different locations have very different inhibitory activity against the growth of *P. larvae* and *A. apis* (Bastos et al., 2008; Wilson et al., 2013). Because propolis is a mixture of resins collected by individual bees, it is likely that the great diversity in the ability of samples of propolis to inhibit the growth of *P. larvae* and *A. apis* is due to the different resins bees collect from various plant species in different regions (Mihai et al., 2012). Therefore, the next step in our study was to explore whether bees change their foraging preference for specific plant resins after challenge with a bacterial or fungal pathogen.

To test if bees alter their selection of resins after colonies are challenged with a bacterial or fungal pathogen (*P. larvae* and *A. apis*, respectively), we collected resin loads from the hind legs of returning resin foragers during each observation (pre- and post-challenge). Individual resin loads were stored in separate glass vials and the botanical source of the resin was further analyzed in the laboratory.

It is difficult to monitor bees foraging for resin on plants because resin foraging is particularly rare, compared to others types of foraging, and bees often collect resin high in the canopy of trees, which makes it difficult to observe resin foraging directly. We can identify the plant source of a resin collected by a bee by chemically comparing the resin loads of returning foragers with resins collected directly from plants. This strategy is very similar to how we track pollen foraging. Since the shapes (morphology) of pollen grains

are characteristic of specific plants, we can use a microscope to match the morphology of bee-collected pollen to the morphology of pollen collected from flowers. Resins have chemistries that are characteristic of specific plants, so we use chemical signatures, rather than morphology, to identify resin sources.

We examined resin chemistries using two scientific techniques in series, liquid chromatography and then mass spectrometry (LC-MS). Essentially, LC-MS sorts the hundreds of compounds found in resins by water solubility and size. This information is then condensed into a graphical "fingerprint". If the chemical pattern, or fingerprint, of a bee-collected resin load is the same as the chemical pattern of a resin collected directly from a plant, we can conclude that the bee visited that specific plant (Figure 1.7).

So far, in our analysis from 2012 and 2014, we found that all bees collected resin from five botanical sources in St. Paul, Minnesota: *P. deltoides* (Eastern conttonwood trees), *P. hybrid* (poplar hybrid trees), and three sources we are in the process of identifying, which we will call unknowns 1, 2 and 3. The majority of bees in all colonies collected resin from the most abundant resin-producing tree in our area, Eastern cottonwood (*Populus deltoides*); while resin from the other sources was not collected in great quantities (Table 1.2).

For the most part, all colonies continued to collect resin from the same sources after they were challenged with either the bacterial or fungal pathogen, with the exception of colonies in 2012 that did not collect resin from hybrid poplar after challenge with *P. larvae*. In general, colonies simply increased the number of foragers collecting resin from the plants they were already visiting.

We measured the antimicrobial activity of the resins bees collected in liquid culture using the same assay as we used to measure the bioactivity of larval food (see 1.3). Of the five different plant sources of resin, we found that the resin from Eastern cottonwood and hybrid poplar have the greatest antimicrobial activity against *A. apis* growth. Resin from Eastern cottonwood also has the highest antimicrobial activity against *P.* larvae, but hybrid poplar and Unknown 1 have relatively low activity to this bacterial pathogen. Thus, after challenge colonies do not appear to change their foraging preference to collect

resins with higher specific bioactivity (they do not forage for "stronger medicines" for a particular pathogen). We know that there are other trees in the area, such as white spruce (*Picea glauca*) that secrete resin with even higher antimicrobial activity against *P. larvae* compared to Eastern cottonwood (Wilson et al., 2013). However, bees apparently do not collect resin from white spruce in our area, as the chemical signatures of our three unknowns did not correspond to white spruce or any other resin-producing plant identified in Wilson et al. (2013).

Bees' decision making process to collect resin from specific sources after chalkbrood and AFB infection could be driven by the abundance of the plant in the area, the abundance of resin produced by particular plants, the ease of collecting resin from particular plants, and/ or the bioactivity of the resin. Resin collection and choice by bees are unstudied areas that require further investigation.

1.5 Recommendation for beekeepers

Our studies clearly show the benefit of a propolis envelope, particularly an envelope naturally constructed by the bees, to bee health and immune system functioning. The collection of resins to construct a natural propolis envelope is performed by a rare subset of the work foraging force. It is estimated that the number of resin foragers is less than 1% of the total number of foragers in the hive but this foraging preference may be influenced by the bees' genetics (Butler, 1949; Page and Fondrk, 1995). Resin collection is partly a genetic tendency and partly a demand-driven process (Martinez and Soares, 2012; Nakamura and Seeley, 2006). How and what they detect inside the nest to determine this need is not clear. We know that when resin foragers encounter rough surfaces and gaps inside the hive, they respond by collecting more resin to seal these cracks in the nest architecture (Simone-Finstrom et al., 2010). Therefore, a colony of bees can be encouraged to build a natural propolis envelope within standard beekeeping equipment by modifying the inner walls of bee boxes. Commercial propolis traps can be

cut to fit the four inside walls of the hive boxes and stapled with the smooth side of the trap facing the wood and the rough side facing the colony. It is recommended to manage colonies using nine frames instead of ten when using this method. If the inside of the bee box is built with unfinished, rough lumber, scraped briskly with a wire brush, or if small grooves are cut in the interior walls of the box, the bees will apply a layer of propolis in the grooves of rough surfaces, forming a natural propolis envelope.

A cautionary note for beekeepers. Our initial experimental design for our study on the effects of the propolis envelope consisted of three treatments: colonies with no extra propolis (control), colonies with propolis envelope, and colonies fitted with a propolis trap on top of the frames of the top box, as is done to collect propolis commercially. Bees from colonies with the propolis traps on top of the frames showed inconsistent, and sometimes higher immune-related gene expression, compared to bees in the propolis envelope and control colonies. Moreover, bees from colonies with a propolis trap on top of the frames had significantly higher levels of virus (DWV) compared to bees in control and propolis envelope colonies in September 2012, May 2013 and May 2014. The presence of high levels of virus has been correlated with colony death and the reduced efficacy of the bee's immune system. We think that the presence of the water-resistant propolis trap throughout the year on top of the colony could have altered the microenvironment of the colony (e.g. increasing humidity levels or affecting air circulation within the nest), leading to favorable conditions for the growth of pathogens and maybe viruses. Thus, it appears that leaving a propolis trap on top of a colony for a long period of time, and especially over the winter, is not beneficial to bee health and is not recommended.

Finally, there is no evidence that bees consume resins or propolis. We do not recommend that beekeepers feed propolis solution to bees. Because of the highly antibacterial and antifungal properties of propolis, it could risk killing the beneficial microbiome in bees' guts that is also so critical to their health and survival.

1.6 Conclusion

Understanding honey bees' natural defense mechanisms allows us to appreciate how resilient honey bees are and to improve our beekeeping practices to enhance their natural behaviors and defenses. The process of domestication of the *Apis mellifera* species by humans using managed hives has interfered with one very important natural defense mechanism of the honey bee colony, the construction of a propolis envelope. Our results strongly indicate that the propolis envelope serves as an external antimicrobial layer around the colony, providing fundamental benefits to adult bees' immunity (see 1.2), greater colony fitness in early spring after the winter (see 1.2), a therapeutic protection to the brood from AFB and chalkbrood disease (see 1.3) and supports nurse bees' ability to induce a strong and effective immune response after infection, resulting in a lower infection load after two months following bacterial challenge (see 1.3). The last section of this chapter (see 1.4) lends more support to the idea that honey bee self-medicate by increasing the number of resin foragers after the colony is infected with chalkbrood, emphasizing the important role of resin collection and propolis deposition on the colony-level defense response .

Given all the evidence provided here, it is important to recognize the significance of the propolis envelope as a crucial component of the nest architecture in honey bee colonies. When searching for an apiary location, beekeepers should take into consideration both flower abundance and diversity, and the presence of resin producing plants within foraging distance from the apiary.

1.7 Figures

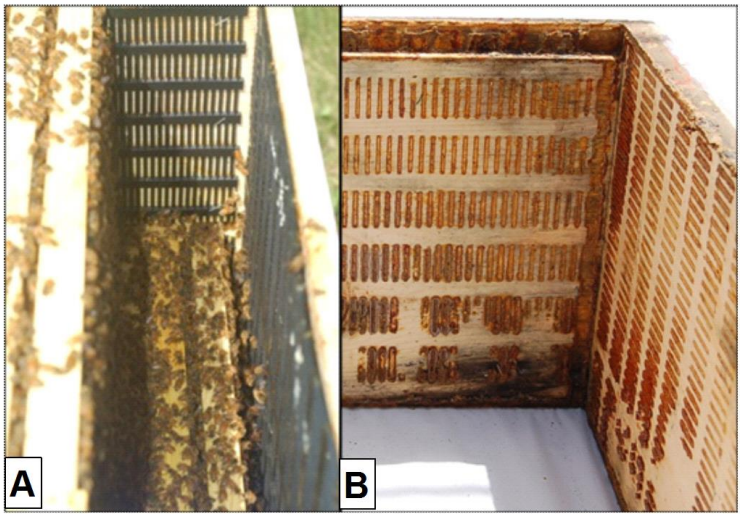

Figure 1.1 Propolis envelope treatment bee box. A) Propolis traps stapled to inside walls of a hive to encourage bees to construct a propolis envelope. B) View of the propolis envelope when traps were removed at the end of the experiment. In each colony, the bees deposited propolis within most of the gaps of each propolis trap (brown lines on the box are the deposited propolis). In a tree cavity, the propolis envelope is contiguous, but bees do not tend to deposit propolis on planed wooden walls in beekeeping equipment, unless lumber is left unfinished.

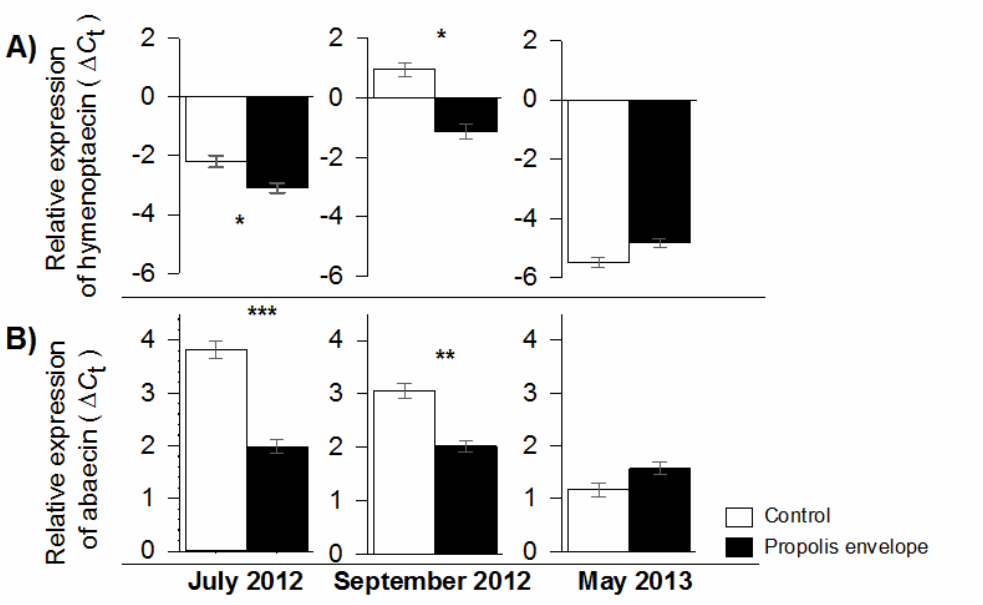

Figure 1.2. Relative expression levels of the immune genes A) hymenoptaecin, and B) abaecin. The expression levels are shown relative to the expression of reference genes Actin and RPS-5, which are not involved in immunity but are produced in relatively equal amounts by bees over their lifetime to regulate other physiological functions. When the immune gene expression is high, the value on the vertical y-axis is a higher number. The height of each bar indicates the average (mean) value of the data for each immune gene, and the lines extending upward and downward from each bar represent the variation, or standard error, around the mean. The white bars represent the control colonies and the black bars represent the colonies with a propolis envelope. Significant differences in gene expression between treatments (when results are considered statistically different) are indicated by * with increasing number of *'s indicated a higher probability of being different: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$). A P value greater than 0.05 indicates that there is no difference between the two treatment groups, while a P value lower than 0.05 means that the two treatments are truly different.

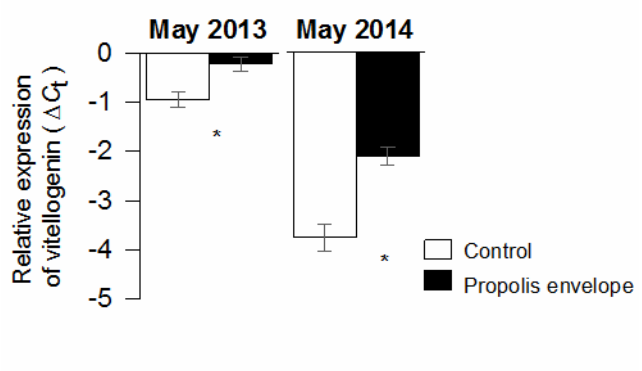

Figure 1.3. Relative expression levels of vitellogenin (mean ± standard error). As in Figure 1, a higher value on the y-axis means higher expression. Vitellogenin levels were higher in bees from propolis envelope colonies (black) compared to control colonies (white), in the spring of both years but only significantly different in 2013. Significant differences between controls and propolis envelope treatment colonies are indicated with * ($P < 0.05$).

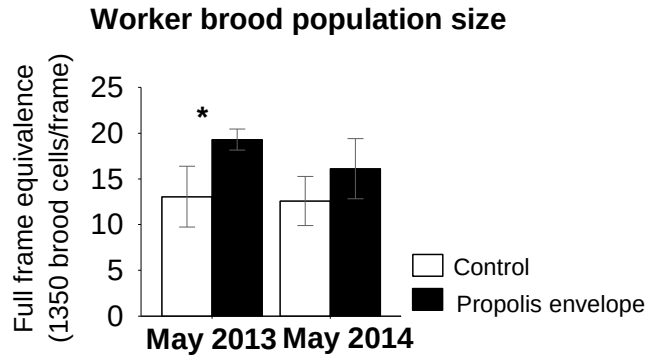

Figure 1.4. Worker brood population size in May 2013 and May 2014. The average (± standard error) number of full frame equivalants (1350 worker brood cells) is presented on the y-axis and the months are indicated in the x-axis. Significant differences between controls (white) and propolis envelope (black) treatment colonies are indicated with * ($P < 0.05$).

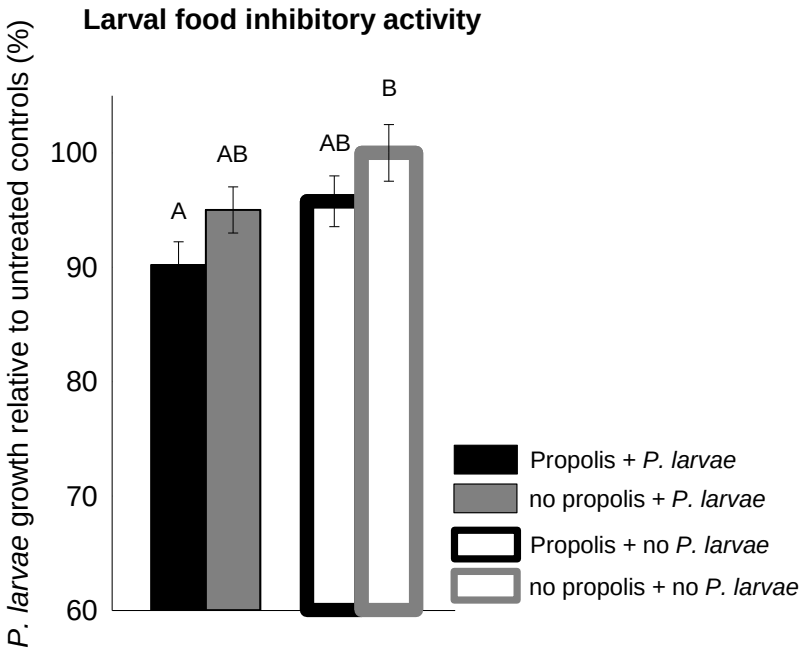

Figure 1.5. A bar graph with standard errors is used to represent the growth-inhibition assay of larval food collected in September, measured as a percent optical density (OD_{600}) relative to untreated controls. Each bar corresponds to a treatment group, and the mean value for each treatment is shown on the y-axis. A low level of *P. larvae* growth (y axis) indicates higher inhibitory activity of larval food. Larval food samples from challenged colonies with a propolis envelope had higher inhibitory activity to *P. larvae* growth relative to untreated controls (lower bacterial growth, y = 83.52%) compared to unchallenged colonies without a propolis envelope. Significant difference among groups, determined by the letters A and B. Treatment groups not connected by the same letter (A or B) are significantly different to each other.

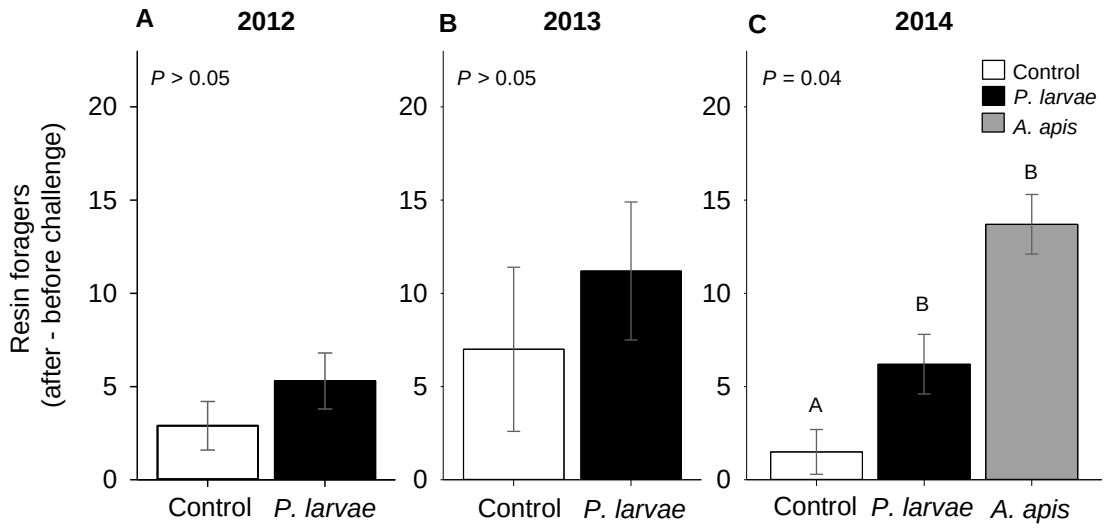

Figure 1.6. Resin foraging activity ± standard error measured as the difference in number of resin foragers after minus number of resin foragers before colony inoculation for A) 2012, B) 2013, and C) 2014. Significant differences between treatment groups was determined by two tailed t-test in 2012 and 2013 and by ANOVA followed by Tukey-HSD test in 2014. Treatment groups not connected by the same letter (A, B) are significantly different.

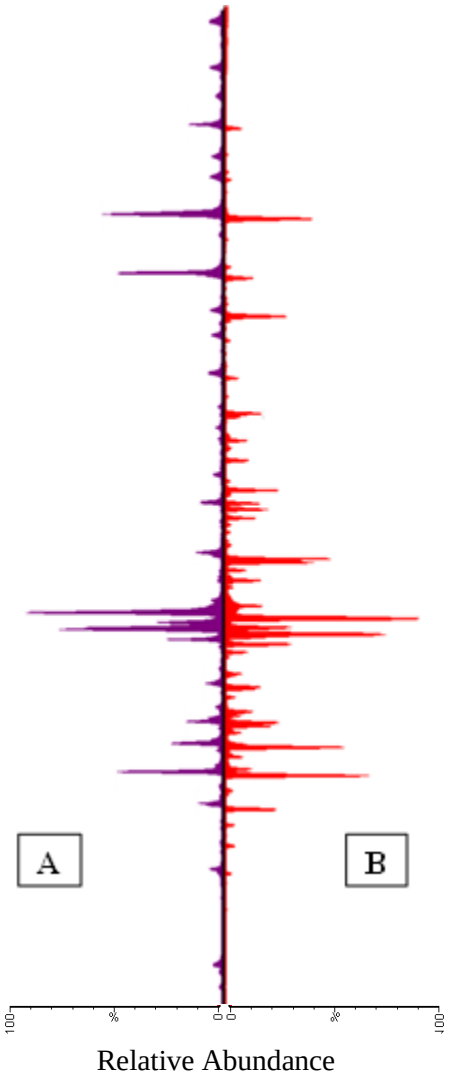

Figure 1.7. Resin fingerprint of Eastern cottonwood trees collected from individual tree buds (A), and fingerprint of resin collected from the bee's hind leg (B). Based on the similarities of the chemical pattern of these two resin fingerprint, we can conclude that the resin collected from this bee is from an Eastern cottonwood tree.

1.8 Tables

Treatment	Number of colonies	AFB clinical sign (mean ± standard error of AFB-infected cells)		
		August	**September**	**October**
No propolis envelope + *P. larvae*	5	6.4 ± 1.6	11.5 ± 2.3	27.3 ± 4.0
Propolis envelope + *P. larvae*	5	4.6 ± 1.4	6.8 ± 2.3	6.7 ± 1.5
Statistical significance		$P > 0.05$	$P > 0.05$	$P = 0.03$

Table 1.1. AFB infection level data was measured by counting the number of cells containing signs of AFB in each comb. The average number of total AFB-infected cells ± standard errors was compared between treatments. A *P* value lower than 0.05 indicates a statistically significant difference between the two treatment groups.

2012	Unchallenged		*Paenibacillus larvae*		*Ascosphaera apis*	
Botanical source	**Before**	**After**	**Before**	**After**	**Before**	**After**
Populus deltoides	5.7 ± 0.8	8.3 ± 0.6	3.8 ± 0.5	8.5 ± 0.7	-	-
Populus hybrid	2.7 ± 0.3	0	2.2 ± 0.3	0	-	-
Unknown 1	0.4 ± 0.2	2.4 ± 0.2	1.4 ± 0.2	3.4 ± 0.3	-	-
Unknown 2	0	1 ± 0.2	0	0.8 ± 0.1	-	-
2014						
Populus deltoides	2.3 ± 0.3	3.8 ± 0.5	2.5 ± 0.5	5.9 ± 0.8	4.2 ± 0.8	16.8 ± 1.2
Populus hybrid	0.1 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	1.3 ± 0.2	1.2 ± 0.2	2.6 ± 0.2
Unknown 1	0.8 ± 0.1	0.8 ± 0.2	0.1 ± 0.1	1.3 ± 0.2	0.2 ± 0.1	0
Unknown 3	1.1 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	0	0

Note. The St. Paul campus of the University of Minnesota is surrounded by urban gardens and landscaping, with diverse species of plants that the bees may be finding and using as resin sources. Until the chemical fingerprints of the resins from the "unknown" botanical sources are identified by other researchers, the unknowns may remain unknown.

Table 1.2. Average number of resin foragers by botanical source per 15-minute observation period (mean ± standard error) in unchallenged (control; 10 colonies/year), *P. larvae* (10 colonies/year) and *A. apis* colonies (10 colonies in 2014 only). Each before and after periods consisted of a total of 12 observations of 15 minutes each.

Chapter 2.

Seasonal benefits of a natural propolis envelope to honey bee immunity and colony health

Summary

Honey bees, as social insects, rely on collective behavioral defenses that produce a colony level immune phenotype, or social immunity, which in turn impacts the immune response of individuals. One behavioral defense is the collection and deposition of antimicrobial plant resins, or propolis, in the nest. We tested the effect of a naturally constructed propolis envelope within standard beekeeping equipment on the pathogen and parasite load of large field colonies, and on immune system activity, virus and storage protein levels of individual bees over the course of a year. The main effect of the propolis envelope was a decreased and more uniform baseline expression of immune genes in bees during summer and fall months each year, compared to the immune activity in bees with no propolis envelope in the colony. The most important function of the propolis envelope may be to modulate costly immune system activity. As no differences were found in levels of bacteria, pathogens and parasites between the treatment groups, the propolis envelope may act directly on the immune system, reducing bees' need to activate the physiologically costly production of humoral immune responses. Colonies with a natural propolis envelope had increased colony strength and vitellogenin levels after surviving the winter in one of the two years of the study, despite the fact that the biological activity of the propolis diminished over the winter. A natural propolis envelope acts as an important antimicrobial layer enshrouding the colony, benefiting individual immunity and ultimately colony health.

2.1 Introduction

Social insect colonies may be considered superorganisms, a group of related individuals living in a nest, whose collective behaviors produce a colony-level phenotype, which in turn influences the behaviors of individuals in the nest (Seeley, 1989). Highly social insects' immune defenses function in a similar collective way: at the individual level, an immune response is initiated via cellular or humoral immune pathways (Evans et al., 2006). At the colony-level, some individuals perform behaviors that defend and protect the colony against pathogens and parasites (Simone et al., 2009). These behavioral defenses in a honey bee colony include hygienic behavior and grooming (Wilson-Rich et al., 2008), antimicrobial secretions (e.g. the spread of venom on bee's cuticle; Baracchi et al., 2011), and the collection of antimicrobial compounds (e.g. resins) from the environment (Simone et al., 2009). Combined with the division of labor among individuals (Naug and Smith, 2007; Stroeymeyt et al., 2014), these behavioral defenses produce a colony-level immune phenotype, or social immunity (Cremer et al., 2007), which in turn impacts the immune response of individuals (Otti et al., 2014).

Behavioral, or social, immunity benefits overall colony health and may have less physiological cost to individuals compared to the cost of maintaining a diverse immune system (Evans and Pettis, 2005; Schmid-Hempel, 2005). In honey bees, social immunity plays an important role in reducing parasite establishment and spread within colonies (Arathi et al., 2000; Evans and Spivak, 2010; Simone-Finstrom and Spivak, 2012).

The collection of antimicrobial resins from the environment by honey bees (Simone et al., 2009) and its deposition into the nest architecture is a fundamental component of their social immunity. Resin is a plant exudate secreted prophylactically to protect young leaf buds from pathogen infection and herbivore attack. It is composed primarily of antimicrobial compounds (e.g. monoterpenes and flavonoids) that play a major defensive role in the survival of the plant (Langenheim, 2003). Honey bees collect plant resins and deposit the resins in the nest as a form of cement, called propolis. When honey bees nest in tree cavities, they use propolis to coat the entire inner surface of the nest cavity, constructing a propolis envelope (Seeley and Morse, 1976). However honey bees do not

construct a natural propolis envelope within standard beekeeping equipment because the inner walls of the wooden boxes are smooth and do not elicit propolis deposition behavior. Instead, bees deposit propolis only in dispersed cracks and crevices and not as a continuous envelope (Simone-Finstrom and Spivak, 2010).

Simone et al. (2009) first tested the benefits of a propolis envelope to the bees' immune system by experimentally coating the inside of boxes with a propolis extract solution (ethanolic solution of propolis) to simulate a propolis envelope surrounding small colonies of honey bees. After just seven days exposure to the propolis enriched nest environment, bees' immune-related gene transcription was significantly lower compared to bees in boxes not enriched with the propolis-extract. The bacterial load (eubacterial 16S gene expression, which measures internal and external bacteria carried by bees) was also significantly lower in bees in propolis-enriched colonies. These results suggested that the propolis reduced the level of immune-elicitors in the nest, so that the bees were able to expend less energy on costly immune system activation (Simone et al., 2009).

Other benefits of propolis to honey bee health have been documented. Numerous *in vitro* studies have demonstrated the inhibitory activity of propolis, and specific compounds within propolis, against the growth of the honey bee bacterial pathogen *Paenibacillus larvae* and *Ascosphaera apis* (Antúnez et al., 2008; Bastos et al., 2008; Bilikova et al., 2013; Lindenfelser, 1968; Wilson et al., 2013; Wilson et al., 2015). It is not known if honey bees actually consume propolis, but Johnson et al. (2012) demonstrated that when bees were experimentally fed propolis in sucrose syrup the transcription of three cytochrome 450s, involved in pesticide detoxification, was induced (Johnson et al., 2006; Mao et al., 2011). The placement of natural propolis in the nest cavity has been positively correlated with brood viability, worker lifespan, honey production, hygienic behavior and pollen stores (Nicodemo et al., 2013; Nicodemo et al., 2014).

Here, we tested the effect of a naturally constructed propolis-envelope within standard beekeeping equipment on the strength, pathogen and parasite load of large field colonies, and immune system activity, virus and storage protein level of individual bees

over the course of a year. Our aim was to examine the relative immune and health benefits of the natural propolis envelope from the scale of the individual bee to the level of the entire colony. At the individual level, we hypothesized that the presence of a propolis envelope enshrouding the nest area would result in a decrease in eubacterial load (based on findings from Simone et al., 2009) and possibly virus load. Additionally, we predicted that in response to the lower level of immune elicitors (pathogens and other microbes) within the nest, the immune-related gene expression in bees from colonies with a propolis envelope would be lower compared to bees in colonies without the propolis envelope (Simone et al., 2009). At the colony level we hypothesized that colonies with a propolis envelope would have greater colony strength (more bees and brood; e.g. Nicodemo et al., 2013; Nicodemo et al., 2014) and would have increased winter survivorship. Our findings revealed significant reduction in the baseline activity of a number of immune gene transcripts in individual bees, but no effects on other measured microbes, pathogens or parasites. Colonies with the natural propolis envelope had increased colony strength after the surviving winter in one of the two years of the study. This is the first study to investigate the seasonal benefits of propolis to honey bees, and demonstrates how the collection and deposition of resins into the nest architecture produces a colony-level immune phenotype that impacts individual immunity, and ultimately colony health.

2.2 Materials and Methods

2.2.1 Stimulation of propolis envelope construction in field colonies

This experiment was conducted at the University of Minnesota Agricultural Experiment Station in Rosemount, Minnesota, United States. Honey bee colonies were established from packages (Nature's Nectar LLC, Minnesota) and were hived in new 10-frame standard Langstroth equipment in mid-April 2012. In mid-April 2013 a second set of colonies was established from packages to serve as a replication of this experiment. In

each replicate, sister queens of Italian derived *Apis mellifera ligustica* were introduced into each colony to reduce genetic variation. Twelve colonies each year were provided with commercially available propolis traps (Mann lake LTD, Minnesota) stapled to the four inner walls of each bee box to encourage the bees to construct a propolis envelope within the nest (propolis envelope treatment; Fig. 2.1). Another twelve colonies each year served as controls; no propolis trap was provided and the bees deposited propolis in the cracks and crevices within the box where they could (control treatment).

2.2.2 Colony management

Honey bee colonies were given routine management and supplemental feeding as needed. Pollen substitute and sugar syrup were provided to new package bees in early spring and additional boxes were added in the summer for honey storage when necessary. No sugar syrup was fed to colonies in the fall and all colonies were left sufficient honey stores to last the northern winter. In September 2012 and 2013, all the colonies were treated to control *Varroa* mites to avoid confounding the effects of the propolis envelope on colony survivorship with the effects of this parasitic mite. In both years, all the colonies were treated with a commercial thymol-based product (Apigard®; Mann lake LTD, Minnesota). In 2013, all colonies still had high levels of *Varroa* (an average of 10.65 mites/100 bees; after the thymol treatment) and, therefore, all received a second miticide treatment with oxalic acid in October (Rademacher and Harz, 2006). No colonies were treated for *Nosema* spp. Colonies were overwintered in Minnesota in the same apiary as they were located during summer and fall and in the boxes according to the treatment they had received.

2.2.3 Colony-level measurements

Colony-level measurements were assessed in the summer (first week of July), fall (last week of September) and following spring (first week of temperatures above 12 ˚C in May). The second replicate year of this experiment did not include colony assessments in

July. All assessments consisted of: 1) Estimating the adult bee population size by counting the number of frames covered with bees for each box (Nasr et al., 1990); 2) Estimating the total amount of worker brood by using a grid (2.56 cm^2) over a frame and counting the number of squares filled with sealed or unsealed brood (Nasr et al., 1990); 3) Collecting a sample of 300 adult bees from brood area in 70% ethanol and quantifying *Varroa* and *Nosema* spp. levels in the laboratory following previous published methods (Lee et al., 2010; Spivak and Reuter, 2001); 4) Inspecting for the presence of clinical signs of diseases in the field (e.g. AFB infected larvae, as noted by the presence of sunken wax capping and uncapped cells containing discolored, ropy brood); and 5) Noting colony survivorship as dead or alive. Colony life-time was recorded as the number of days from the first day of the experiment (day 1) until the inspection date when colony was found dead, or until the last day of the experiment for colonies that did not die.

2.2.4 Sample collection of bees for gene expression analysis

During the colony assessments, newly emerged bees (noted by their location near emerging pupae and their fuzzy appearance; Human et al., 2013), were painted using enamel paint markers and 20 bees per hive were collected after six days. The marked, 7-day old bees were stored in -80 ˚C freezer until analysis. Immunocompetence in bees increases from emergence to day 7-8 of adult life, at which time it is thought that their immune system is fully capable of starting an immune response (Wilson-Rich et al., 2008). We sampled 7-day old bees because immune activity becomes more variable after eight days until bees become foragers, when immunity is highly decreased (Amdam et al., 2005; Simone et al., 2009).

We analyzed the gene expression level of all the following measures using real-time PCR. For the immune response, we measured four antimicrobial peptides (hymenoptaecin, abaecin, defensin-1 and defensin-2), the NF-kB transcription factor of the IMD pathway (relish), and phenoloxidase. We estimated the bacterial loads of the colony by measuring the gene expression of the eubacterial 16S gene (16S rRNA) in

individual bees (interior and exterior bacteria carried by bees) using a universal primer (Evans et al., 2006; Simone et al., 2009) to test for the effect of propolis on bacterial level. The expression of vitellogenin (Vg) was measured as a marker of nutrition status, as it is the main storage protein for bees and a precursor for other proteins. Additionally, Vg is also used by young adult bees (5-16 days old) during the synthesis of the antimicrobial secretion royal jelly, which they use to feed queens and young larvae (Amdam et al., 2003). Finally, we measured levels of three most common viruses in honey bee colonies: Deformed Wing Virus (DWV), Israeli Acute Paralysis Virus (IAPV) and Black Queen Cell Virus (BQCV).

2.2.4.1 Real-time PCR methods

Total RNA was isolated from individual bee abdomens of 7-day old marked bees using TRIzol reagent (Ambion, Austin, TX) following the manufacture's protocol. Quality and quantity of total RNA was measured using a NanoDrop2000 instrument (Thermo Scientific Inc., Grand Island, NY) and 3.5 µg of each sample was used for cDNA synthesis. Prior to cDNA synthesis, RNA was treated with DNAse I (Ambion) and reverse transcription for cDNA synthesis was carried out using Superscript II (Invitrogen, Grand Island, NY). Complementary DNA was diluted 1:3 with RNAse and DNAse free water. Relative quantification of viral levels, candidate genes used for the immune system response, bacterial loads and blood storage protein (Vg), were analyzed via real-time PCR (Bio-Rad CFX96). Samples for real-time PCR were prepared using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Primer sequences used for this experiment were selected from the literature when available and optimal annealing temperature was met (Table 2.1). Otherwise, primers were designed using MacVector version 12.5.1 and specificity was confirmed using primerBLAST.

2.2.5 Seasonal antimicrobial activity of propolis

Honey bees in Minnesota do not forage for resin (or for any resources) from October to April. Therefore, we tested if the resin deposited within the hive in September maintained its bioactivity over the winter, until April of the following year. A high throughput bacterial growth assay was performed to assess the inhibitory activity of propolis on the growth of the honey bee bacterial pathogen *Paenibacillus larvae*. A total of nine propolis samples were collected from the traps of three propolis envelope treatment colonies in September and in the following April. Three colonies from the propolis envelope treatment were randomly selected and three propolis samples (3-5 g each) were collected from each colony by detaching the traps from the wall and scraping the propolis using a hive tool. Bioactive compounds of propolis (approximately 1g of each propolis sample) were extracted in 1 ml of acetonitrile. Propolis extract concentrations (wt/vol) were calculated by air drying a 200 µl aliquot of the propolis acetonitrile extract using a speedvac concentrator and dividing the precipitates weight by the initial volume of 200 µl. *Paenibacillus larvae* (from stock strains obtained from the USDA Agricultural Research Service culture collection, NRRL# B-2605) were cultured in brain/heart infusion broth and the bacterial growth assay was conducted following Wilson et al. (2015) methods. Propolis extracts were diluted in acetonitrile to final concentrations ranging from 175 mg/l to 8 mg/l, transferred into 96 well plates, dried under nitrogen gas, and resolubilized in 100 µL of brain/heart infusion broth for 15 min. *P. larvae* liquid culture was transferred into the well plates (creating a 1:100 dilution of *P. larvae* in each well), and well plates were incubated at 37°C and 400rpm for six hours. Bacterial growth inhibition was evaluated in 96-well plates by measuring turbidity (optical density at time 0 h subtracted from time 6 h, OD_{600}) of treated cultures relative to untreated controls using a microplate spectrophotometer.

2.2.6 Statistical analysis

2.2.6.1 Colony-level data analysis

Colony-level measurements (adult population size, worker brood size, *Varroa* and *Nosema* levels) were compared between treatment groups (propolis-envelope colonies

and control colonies) using two tailed t-tests, using R version 2.15. Colony status was recorded at each hive inspection as dead or alive. For colonies found dead after the winter, the date of death was recorded as the first spring inspection day, as no inspections of the colonies were made from November through April. Colony survivorship was analyzed using the nonparametric Kaplan-Meier method and Wilcoxon Rank-sum test, using JMP® software (Cary, NC), to test differences between groups.

2.2.6.2 Gene expression data analysis

C_t values were collected based on the default Bio-Rad CFX threshold search criteria. The relative expression of target genes was normalized to the average of two reference genes ($\Delta Ct = (\overline{x}\ (\text{reference genes})\ Ct - \text{target gene}\ Ct)$) and an F test (ANOVA) was performed using R version 2.15, with colony as a random factor and treatment group as a fixed effect.

2.2.6.3 Seasonal variability in gene expression

Seasonal variability of the immune gene expression was obtained as the standard deviation of all ΔC_t values for each gene separately. The relative expression for each gene was combined for all sampling periods (e.g. from July 2012 to May 2013 for the 2012-2013 experimental year and September 2013 - May 2014 for the 2013-2014 experimental year) and the difference in variability was compared between treatment groups by the Levene test using R version 2.15.

2.2.6.4 Bacterial inhibition assay

An IC_{50} for the propolis inhibition assay was calculated by fitting a four-parameter logistic equation to the sigmoidal inhibition curves using Systat software version 12.5 (San Jose, CA). The IC_{50} values of propolis samples from September and April was compared using a two tailed t-test using R version 2.15.

2.3 Results

2.3.1 Effects of propolis on colony-level measurements

The experiment was replicated over two years. The first set of 24 colonies (12 with propolis envelopes, 12 without) was followed from June 2012 to early May 2013. A second, new set of 24 colonies was followed from September 2013 to early May 2014.

Colony strength: Brood areas were similar between the treatment groups during the summer and fall inspections each year. By the following spring of the first year, May 2013, colonies with a propolis envelope had significantly more worker brood compared to the control colonies ($t_{(12)}$= 2.19, two-tailed P = 0.04), but this positive trend was not significant in the second replicate of the experiment by May 2014 (Fig. 2.2a). There were no significant differences in the population size of colonies (adult worker bees) in the summer, fall or spring, in both replications of the experiment (Fig. 2.2b).

Colony survivorship: Colony survivorship (colony life time from April until May of the following year, measured in days) was significantly higher in colonies with a propolis envelope compared to control colonies in the 2012-2013 experimental year (P = 0.04; Fig. 2.3). Colony survivorship was the same between groups in the following experimental year, 2013-2014.

Parasite and pathogen levels: As expected, because mite levels were controlled during fall treatments, there was no significant difference in levels of *Varroa* mites between treatment groups before or after fall treatment (thymol-based and oxalic acid), and by the following spring in either replication of the experiment (Fig. 2.4a). In September of 2012, the colonies with a propolis envelope had lower levels of *Nosema* spp. but the difference was only marginally significant ($t_{(16)}$ = 1.84, two-tailed P = 0.08). Overall, all levels of *Nosema* spp. were generally below 1 million spores/bee in both years, and no colonies received treatments for *Nosema* spp. (Fig. 2.4b).

2.3.2 Effects of propolis on virus

There was no significant difference in levels of all three viruses (DWV, IAPV and BQCV) in bees from colonies with the propolis envelope compared to bees from control colonies in the first replicate (samples from September 2012, and May 2013), and in fall of the second replicate, September 2013. In May 2014, bees from colonies with a propolis envelope had lower levels of BQCV, but the difference was only marginally significant ($F_{1,11}$= 3.45, P = 0.09; Fig. 2.5).

2.3.3 Effects of propolis on individual bee immune system

Summer and fall: The transcription levels of two of the six immune gene transcripts, hymenoptaecin and abaecin, in bees from colonies with a propolis envelope were significantly lower compared to bees from control colonies in July 2012 ($F_{1,16}$ = 5.77, P = 0.03; $F_{1,16}$ = 20.76, P = 0.0003; respectively; Fig. 2.6a).

By September 2012 (Fig. 2.6b), all six measured gene transcripts for the immune system response (hymenoptaecin, abaecin, defensin-2, defensin-1, relish and phenoloxidase) were expressed at significantly lower levels in bees from the propolis envelope treatment compared to bees from the control colonies ($F_{1,16}$ = 5.98, P = 0.03; $F_{1,16}$ = 11.14, P = 0.004; $F_{1,16}$ = 6.29, P = 0.02; $F_{1,16}$ = 12.04, P = 0.003; $F_{1,16}$ = 35.39, P < 0.0001; $F_{1,16}$ = 14.16, P = 0.002; respectively). In September 2013, in the second replicate of the experiment, bees from the propolis envelope colonies had significantly lower levels of hymenoptaecin and abaecin (Fig. 2.6d) compared to bees from the control colonies ($F_{1,9}$ = 5.71, P = 0.004; $F_{1,9}$ = 7.82, P = 0.02; respectively). Samples were not collected in July 2013.

Spring of the following year: In the first replicate, by May 2013, bees in the propolis envelope treatment showed significantly higher transcription of three immune-related genes, defensin-1, relish, phenoloxidase ($F_{1,9}$ = 24.18, P = 0.0006; $F_{1,9}$ = 14.90, P = 0.004; $F_{1,9}$ = 11.06, P = 0.007; respectively; Fig. 2.6c), and there were no differences in

levels of the other immune gene transcripts. In May 2014, there were no differences between bees in the treatment groups for any of the immune transcript levels (Fig. 2.6e).

2.3.4 Effects of propolis on the seasonal variation of the immune response

Variability in immune gene expression was obtained as the standard deviation of all ΔC_t values (summer, fall and spring pooled together) for each gene separately. The relative expression for each gene was combined for all sampling periods (e.g. from July 2012 to May 2013 for the 2012-2013 experimental year and September 2013 - May 2014 for the 2013-2014 experimental year) and the difference in variability was compared between treatment groups by the Levene test. From July 2012 to May 2013, there was significantly less variation in gene expression of five immune genes (hymenoptaecin, abaecin, defensin-1, defensin-2 and relish) in bees from colonies with a propolis envelope compared to bees from the control colonies. In contrast, there was significantly higher variation in levels of phenoloxidase over that season in bees from colonies with a propolis envelope compared to bees in control colonies. In the second replicate of the experiment, from September 2013 to May 2014, bees from colonies with a propolis envelope had significantly lower variation in hymenoptaecin, abaecin, defensin-1, defensin-2 and phenoloxidase gene expression levels. One of the genes, relish, was only marginally significantly lower compared to bees from control colonies (Table 2.2). Thus in general, the seasonal variability of the immune gene expression was lower in the sample population from colonies with a propolis envelope compared to the sample population from control colonies.

2.3.5 Effects of propolis on eubacterial levels

Levels of general eubacteria were similar between treatment groups in July 2012 and in the fall and spring of both replicate years (Fig. 2.7).

2.3.6 Effects of propolis on vitellogenin levels

Vitellogenin gene expression was measured as a marker of bee nutritional status. In the first replicate, Vg was expressed at similar levels between treatment groups in July 2012, and by September 2012, bees from the propolis envelope treatment had significantly lower levels of Vg compared to bees from the control colonies ($F_{1,16}$ = 10.23, P = 0.005; Fig. 2.8a). In September 2013 no significant difference in the expression of Vg was observed between treatment groups (Fig. 2.8b). By May of both years, 2013 and 2014, bees in the propolis envelope treatment showed significantly higher transcription of Vg ($F_{1,9}$ = 9.21, P = 0.03; $F_{1,9}$ = 8.07, P = 0.02; respectively; Fig. 2.8a,b).

2.3.7 Seasonal antimicrobial activity of propolis

Honey bees in Minnesota do not forage for resin (or for any resources) from October to April. Therefore, we tested if the resin deposited within the hive in September maintained its bioactivity over the winter, until April of the following year. Propolis samples collected from within colonies in October had higher inhibitory activity (significantly lower IC_{50} value, 87 mg/l) against the bacterial pathogen *P. larvae,* compared to propolis samples from the same colonies the following April. Figure 2.9 shows that propolis samples collected in April had significantly lower inhibitory activity against *P. larvae* growth (IC_{50} = 207 mg/l; $t_{(4)}$ = 3.54, P = 0.02).

2.4 Discussion

This is the first study of the seasonal effects of a natural propolis envelope on the immune system of honey bees. Our results from summer and fall extend those of Simone et al. (2009), who reported a decrease in two immune-related genes' expression in bees after only seven days exposure to propolis-extract solution experimentally coated inside

the walls of small colonies. Organic solvent extracts of propolis may not contain all active compounds, and is not how bees are exposed to propolis naturally, thus we allowed the bees to construct their own propolis envelope. We found the natural propolis envelope served to lower immune gene transcription in individual bees over the summer and fall months. The immune system is one of the most costly physiological systems to maintain in animals (Evans and Pettis, 2005; Schmid-Hempel, 2005). Therefore, a decrease in energetic costs associated with the maintenance of an up-regulated immune system will help bees to allocate their energy to perform vital tasks (e.g. foraging, rearing brood) and to maintain higher storage protein levels required for overwintering success. After winter, before the bees were actively collecting resin again, we found the propolis within the nest had lost much of its antimicrobial activity from the previous fall. Correspondingly, there were no significant differences between bees from the two treatment groups in transcript levels of most immune genes in May 2013 and 2014, with the exception of three genes (defensin-1, relish and phenoloxidase), which were significantly higher in May 2013 in bees from colonies with a propolis envelope. The presence of a natural propolis envelope within the nest corresponded to greater colony survivorship in the first replicate year and greater brood area in the spring of 2013. There were no differences in brood area between groups in May 2014, but Vg levels, an indicator of nutritional health, were significantly higher in both May 2013 and May 2014 in bees from colonies with a propolis envelope compared to bees from control colonies. The levels of pathogens, including viruses and parasitic mites (*Varroa destructo*r), did not differ between colonies in the two treatment groups. In contrast to previous findings (Simone et al., 2009), eubacterial 16S gene expression did not differ in bees from colonies with a propolis envelope or without. It was previously hypothesized that the propolis benefited the honey bee immune system indirectly, first by lowering the amount of microbes within the nest and subsequently lowering immune gene transcription (Simone et al., 2009). Our current findings suggest that the propolis envelope may have an additional direct effect on the immune system.

Honey bees have several layers of defense mechanisms: the individual immune system response, behavioral immune defenses (e.g. hygienic behavior or grooming), antimicrobial secretions (e.g. royal jelly and venom), and the collection of antimicrobial

compounds (resin) from the environment (Evans and Spivak, 2010). The active collection and deposition of antimicrobial plant resins, or propolis, in the nest architecture produce a colony-level immune phenotype, or social immunity (Cremer et al., 2007; Simone et al., 2009). The physical presence of a propolis envelope in the nest architecture is an additional layer of defense for the colony; it is an external antimicrobial barrier that has a direct effect on the baseline expression of immune-related genes of individual bees. The propolis envelope may be considered as an environmentally derived component of the bee's defense mechanism.

The insect immune system is comprised of both humoral and cellular immune responses. The humoral immune response includes the biosynthesis of antimicrobial peptides (AMPs) via signaling pathways (Toll, IMD, Jak-STAT; Evans et al., 2006). Cell-mediated immune responses involve hemocyte-associated defenses. These cellular defense mechanisms include phagocytosis, encapsulation and nodulation, which are often followed by a cellular-associated response of melanization via the activation of the phenoloxidase cascade in hemocytes (Söderhäll and Cerenius, 1998; Strand, 2008). Our study demonstrates that the effect of propolis on the honey bee immune system occurs both on humoral immunity (AMPs expression) and on cellular immunity (phenoloxidase activation cascade). Two AMPs (hymenoptaecin and abaecin) were consistently low in bees from the propolis envelope treatment during summer and fall in 2012 and fall of 2013. Additionally, defensin-1, defensin-2, relish and phenoloxidase showed significantly lower expression in September 2012. The same trend was present in September 2013 for defensin-1, defensin-2 and phenoloxidase, although not significantly different. It remains to be determined if a few key genes play a more important role in honey bee immunity than others (e.g. AMPs vs. phenoloxidase); although it has been hypothesized that it is less costly for insects, under high risk of infection (such as in social insect nest environments), to invest in AMP synthesis compared to maintaining the phenoloxidase cascade active (Moret, 2003). If verified, it could explain the consistently higher expression of hymenoptaecin and abaecin in July and September 2012, and September 2013 in bees from control colonies compared to bees from colonies with a propolis envelope.

The direct effect of propolis on immune cells of vertebrates has been well studied (reviewed in Sforcin, 2007). Propolis has been shown to increase macrophage microbicidal activity (Salomão et al., 2004), enhance the lytic activity of lymphocytes (Kaneno, 2005), and decrease lymphoproliferation (Sá-Nunes et al., 2003) in mice and humans, *in vivo* and *in vitro* respectively. The mode of action by which propolis may regulate immune gene expression in honey bees is unknown. It is possible that, similar to vertebrates, propolis increases cellular immune responses and indirectly decreases the activation of the humoral immune response cascade. Our study did not assess the antimicrobial activity of propolis on bee hemocytes directly. Further investigations will contribute to a better understanding of the immune-modulatory mode of action of propolis on social insects' immune systems.

We found a decrease in the propolis inhibitory activity on the growth of *P. larvae* in the samples collected in the spring compared to propolis samples from the previous fall. These results suggest that propolis loses its bioactivity over the winter, when collection of resin ceases until plant sources of resin have new growth and produce new resin when environmental temperatures are favorable. As a result, it is plausible to assume that in the spring of the following year, the direct effect of propolis on the immune system of honey bees is minimal, if any. The transcription of defensin-1, relish and phenoloxidase was significantly higher in bees from colonies with a propolis envelope compared to bees in control colonies in May 2013 but not in May 2014. We do not have a clear explanation of why these three immune genes were significantly higher in May 2013 in the propolis envelope treatment group. Little is known about the immune system response in spring bees compared to summer and fall bees. Future research exploring the baseline expression of bees' immune genes in the spring would greatly contribute to our understanding of the natural seasonal variation of the immune system response.

There was a significant seasonal variation in immune gene transcription from summer to the following spring in 2012-2013 and from fall to the following spring in 2013-2014. Dawkins et al. (2013) support the hypothesis that an important indicator of a healthy population is represented by more uniformity, or low variance, in the health-

related measures. Unhealthy individuals contribute to a wider spread of the population data while healthy populations present a more narrow range of results (Dawkins et al., 2013). Here we found that in both years of this study, the seasonal variation in gene transcription was significantly lower, and thus more uniform, in bees from the propolis envelope treatment colonies for the majority of the genes analyzed, potentially representing a healthier population. It may be that the most important function of the propolis envelope is to modulate costly immune system activity.

Our original hypothesis was that eubacterial load (as measured by 16S rRNA gene expression) would be lower in propolis envelope colonies based on previous findings of Simone et al. (2009). However, our results showed no significant differences in eubacterial gene expression between the groups in either replicate year. Although the main research interest of our experiment and Simone et al. (2009) were similar, these two studies differed on the size of experimental colonies, and the duration of the experiment and type of propolis (extract vs. natural), which could have led to slightly different results. Nonetheless, both studies found that bees in propolis-rich colonies had a lower immune gene expression compared to bees in propolis-poor (control) colonies. The lack of difference of pathogen and bacteria levels between treatment groups, but a significant decrease in immune gene expression in bees from propolis envelope colonies suggests a direct effect of propolis to bees' immune system. The 16S ribosomal RNA sequence is highly conserved among bacteria species (Stackebrandt and Goebel, 1994), and thus a primer designed for this gene will bind to most bacterial DNA present in the honey bee (pathogenic, beneficial, commensals and fortuitous). Given that honey bees have a large number of bacterial symbionts located in the honey stomach (Olofsson et al., 2014), our results may represent the level of not only pathogenic but also beneficial bacterial strains. Future studies investigating specific bacterial strains will be needed to elucidate the effect of the propolis envelope on honey bee microbiota and pathogenic microbes.

There is strong evidence that RNA interference (RNAi) plays a major role in honey bees' defense against viral infections (Desai et al., 2012; Flenniken and Andino, 2013; Maori et al., 2009). However, there is contradictory evidence concerning the role of

humoral immunity to combat viral infection. Azzami et al. (2012) reported that antimicrobial peptides (e.g. hymenoptaecin and abaecin) expression is not altered upon viral challenge, while a more recent study showed that IAPV infection up-regulates multiple immune signaling pathways in adult bees (Chen et al., 2014). The lack of significant difference in viral level between treatments, in both replicate years, strongly indicates that differences in immune system activity observed in our study are not due to viral infection. The propolis treatment did not appear to have antiviral activity (except marginal activity only for BQCV in May 2014), although it has been reported that some viruses are more susceptible to propolis than others *in vitro* (Amoros et al., 1992; Kujumgiev et al., 1999). The antiviral activity of propolis against human viruses is well documented in human cell culture (Amoros et al., 1992; Gekker et al., 2005; Kujumgiev et al., 1999; Schnitzler et al., 2010). Viruses are obligate intracellular parasites and they must enter host cells in order to live and reproduce. Schnitzler et al. (2010) suggested that the chemical compounds of propolis decrease HSV-1 viral infection *in vitro* by binding to important viral proteins responsible for the adsorption or entry of the virus into the host cell. Additionally, pre-treatment of herpes virus with propolis prior to infection increased the antiviral effect of propolis *in vitro* (Amoros et al., 1994; Schnitzler et al., 2010). One common viral infection route in honey bees is via ingestion of pathogen-contaminated food resources (Chen et al., 2006). It is not known if bees ingest propolis, or if bees add propolis to food materials stored in combs (e.g. pollen, honey). If they do, viruses might come into contact with propolis prior to infection (Simone-Finstrom and Spivak, 2010). Further studies will be necessary to understand the mode of action of propolis against intracellular parasites, such as viruses and *Nosema* spp.

Our study aimed to investigate the effects of honey bees' natural defense mechanism under normal field conditions. Thus, although pathogen levels were similar between treatment groups each year, differences in the intensities of natural occurring pathogens and parasites occurred between years. Levels of *Varroa* mites and DWV were significantly higher in September 2013 compared to September 2012 and *Varroa, Nosema* spp. and BQCV levels were significantly higher in May 2014 compared to May 2013 (Table 2.3). In general, higher levels of parasite, pathogen and virus were detected

in colonies during the 2013-2014 study year, which could have contributed to the presence of slightly different patterns of gene expression levels between replicate years.

Although there is evidence that propolis has activity against the parasitic mite *Varroa destructor* (Damiani et al., 2010; Garedew et al., 2002), the bioactivity of propolis was observed only in laboratory conditions and we did not note any effect of the propolis on *Varroa* levels in the field. Lack of significant difference on the levels of *Varroa* mites in May between control and propolis envelope colonies was expected as all colonies received miticide treatment before the winter. The seasonal dynamics of *Varroa* infection intensities was in accordance with previous studies (Rosenkranz et al., 2010), with rising levels of *Varroa* mites from summer to fall.

At the colony level we found that the presence of a natural propolis envelope within the nest corresponded to greater colony survivorship at the end of the first experimental year but not in the second year. In the first replicate year, four colonies from the control treatment experienced a sudden decline in the summer (two colonies in June and the other two in July) and one colony from each treatment group died before the winter. The cause of death of these colonies was undetermined. Although the number of colonies lost during the winter in both replicate years was similar between treatments, overall survivorship was significantly higher in the first replicate year in the group of colonies that had a propolis envelope. Additionally, we found that colonies with a propolis envelope had greater brood areas in the spring of one year and slightly, but not significantly more brood in May 2014. These results are supported by Nicodemo et al. (2014), who found that high-propolis producing colonies had significantly more brood compared to low-propolis producing colonies. We also found that bees from the propolis envelope colonies had significantly higher levels of Vg in May 2013 and 2014. The high Vg levels in bees from propolis envelope colonies in the spring of both replicate years suggest that these bees had more protein storage in the spring compared to bees in control colonies and, therefore, were able to rear more brood than control colonies. Vitellogenin level is a good marker of nutrition status; it is the main storage protein for young bees (approximately 40% of total protein present in the hemolymph; Engels et al., 1990) and a

precursor for other proteins (Amdam et al., 2003; Amdam et al., 2004). It has also been shown that young worker bees, performing the task of feeding young larvae, use Vg during royal jelly synthesis (Amdam et al., 2003). The amount of Vg in bee hemolymph is positively influenced by the quantity of pollen ingested by bees (Bitondi and Simoes, 1996) and colonies with higher amounts of pollen rear more worker brood in the spring compared to colonies with low pollen or pollen substitute (Mattila and Otis, 2006). The transcription of Vg was significantly higher in September 2012 in bees from control colonies. The immune gene expression data suggests that bees from the control treatment invested more in immune functions than bees from the propolis envelope group in September 2012. Therefore, it is possible that the significant high level of Vg in bees from control colonies in September 2012 is linked to its role in honey bee immunity as a potent zinc carrier and zinc-binding protein and not as a nutritional marker (Amdam et al., 2004).

2.5 Conclusion

This study provides evidence that the incorporation of resin from the environment into the nest architecture in the form of a propolis envelope can benefit honey bees at the colony and individual level. Our results suggest that a propolis envelope within the hive benefits colony strength (e.g. increasing worker brood population) in the spring, which could largely benefit the colony at this crucial time in their life-cycle. We also found that the presence of a propolis envelope increased colony survivorship in one year of the study, and directly affected individual health (e.g. decreasing the baseline expression on immune-related genes in the summer and fall and maintaining a less variable immune system function). Promoting honey bees' natural defenses by investigating the general and specific benefits of propolis may lead to novel and sustainable ways to improve bee health and mitigate some losses.

2.6 Acknowledgments

We would like to thank Gary Reuter, from the University of Minnesota, for assistance with honey bees' colony management. Dr. Brad Mogen for assistance with honey bee viral quantitative PCR analysis and the contribution of undergraduate students of the BEE course (Bees Enhancing Education) at the University of Wisconsin, River Falls to this study during their research training. We also acknowledge the support of all the members of the Bee Lab at University of Minnesota.

2.7 Figures

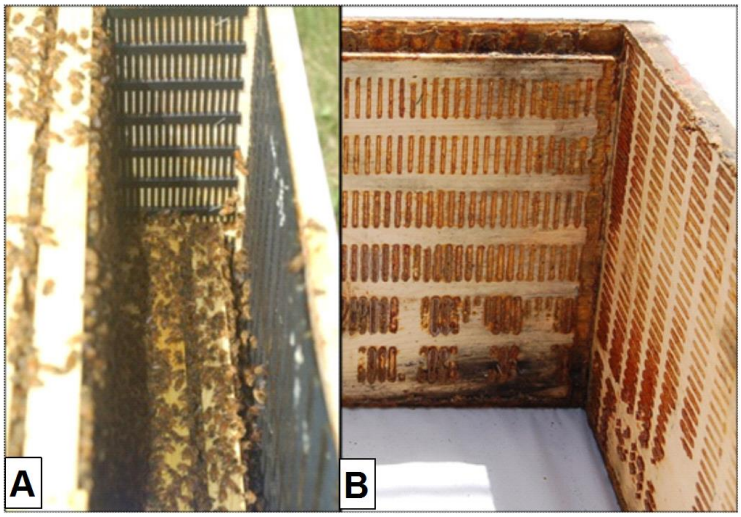

Figure 2.1. Propolis envelope treatment box. A) Propolis traps stapled to inside walls of a hive to encourage bees to construct a propolis envelope. B) View of the propolis envelope when traps were removed at the end of the experiment. In each colony, the bees deposited propolis within most of the gaps of each propolis trap (brown lines on the box are the deposited propolis). In a tree cavity, the propolis envelope is contiguous, but bees do not tend to deposit propolis on planed wooden walls in beekeeping equipment, unless lumber is left unfinished.

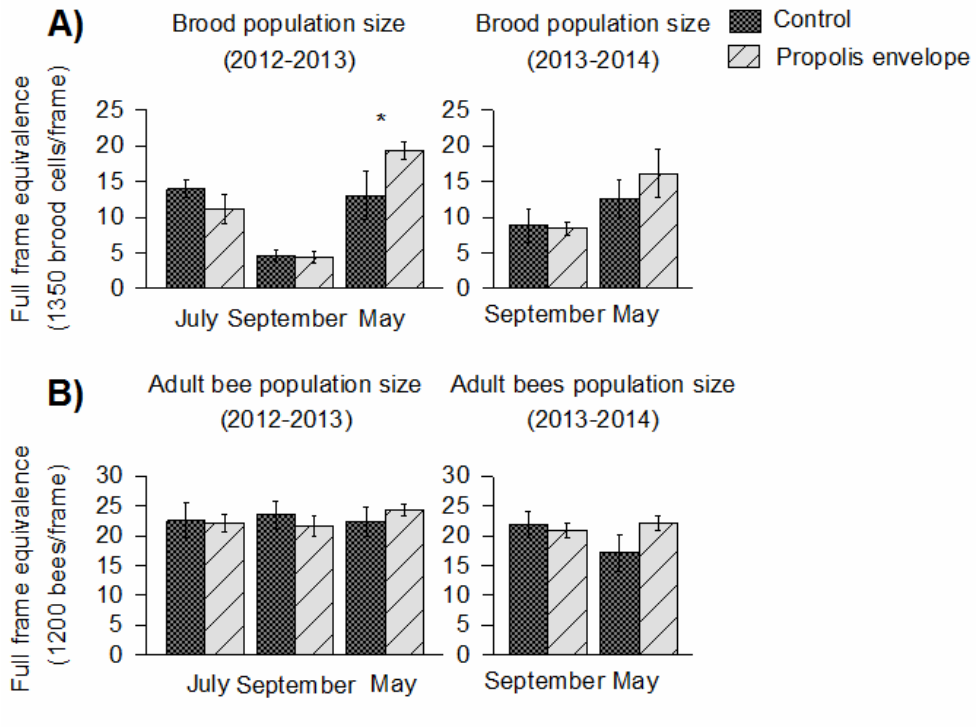

Figure 2.2. Colony strength measurements show that the presence of a propolis envelope benefits colony strength in early spring. Average ± SEM of full frame equivalents for A) brood population size (1350 worker brood cells/frame) and B) adult bee population size (1200 adult bees/frame), for the months of July 2012 (N = 19 colonies), September 2012 (N = 17 colonies), May 2013 (N = 14 colonies), September 2013 (N = 24 colonies) and May 2014 (N = 16 colonies). Significant differences between controls and propolis envelope treatment colonies are indicated with * = $P < 0.05$.

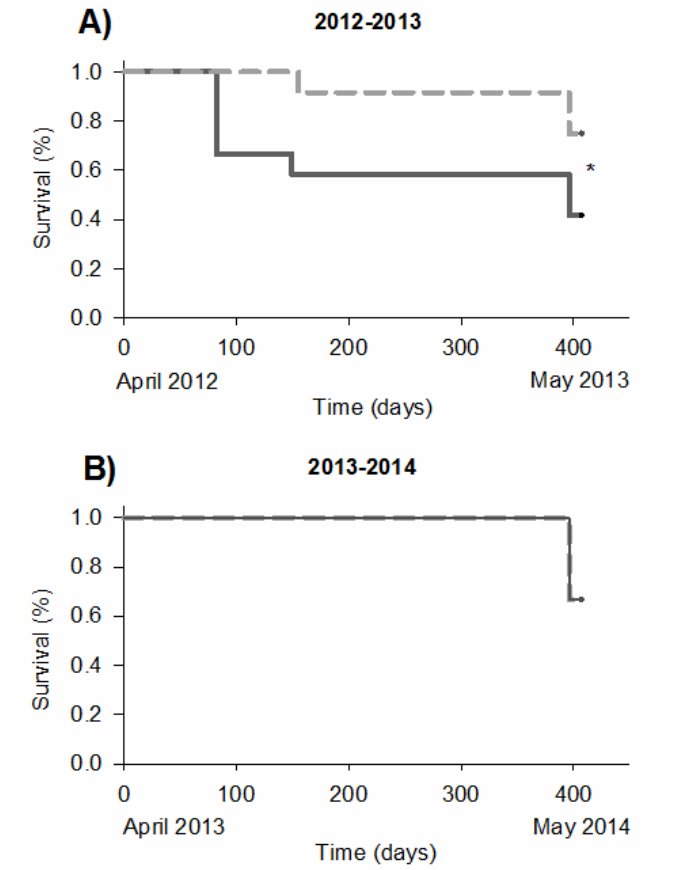

Figure 2.3. Kaplan-Meier survivorship curve comparing survival (proportion of colonies alive) for all control colonies (solid gray) and colonies with a propolis envelope (dashed gray). (A) 2012-2013 experimental year and (B) 2014-2015 experimental year. Twelve colonies for each treatment were used in both replicates at the beginning of the experiment. Significant difference between groups, determined by Wilcoxon Rank-sum test, indicated by * = $P < 0.05$.

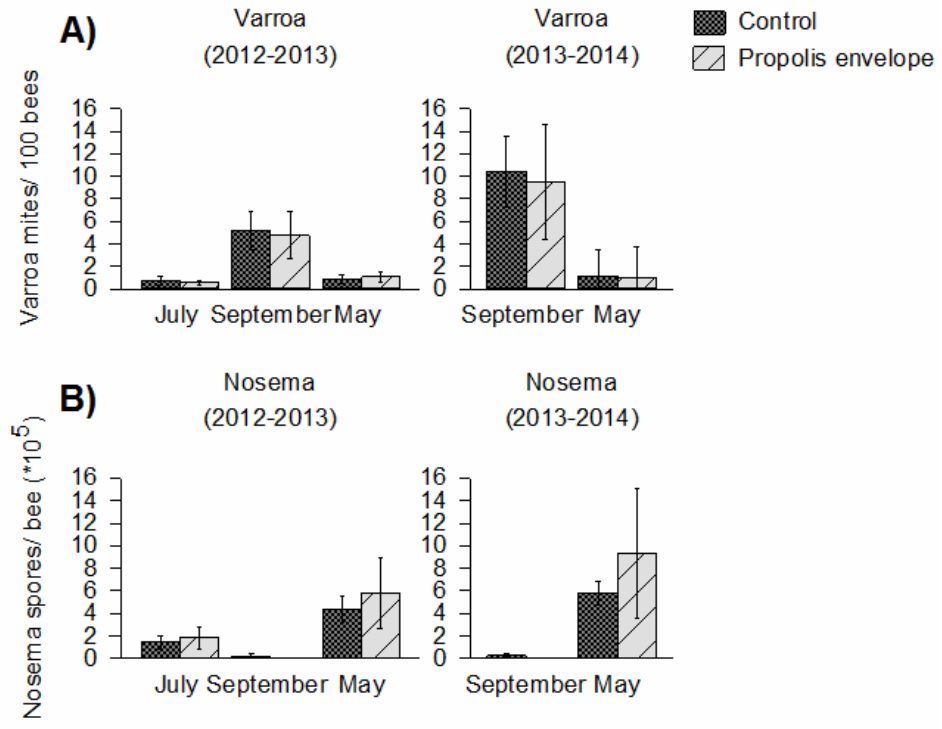

Figure 2.4. Infestation levels average ± SEM (12 colonies per treatment) of parasites (*Varroa* mites) and pathogens (*Nosema* spp.). A) Number of parasitic *Varroa destructor* mites per 100 bees and B) Number of *Nosema* spp. spores per bee for the months of July 2012 (N = 19 colonies), September 2012 (N = 17 colonies), May 2013 (N = 14 colonies), September 2013 (N = 24 colonies) and May 2014 (N = 16 colonies).

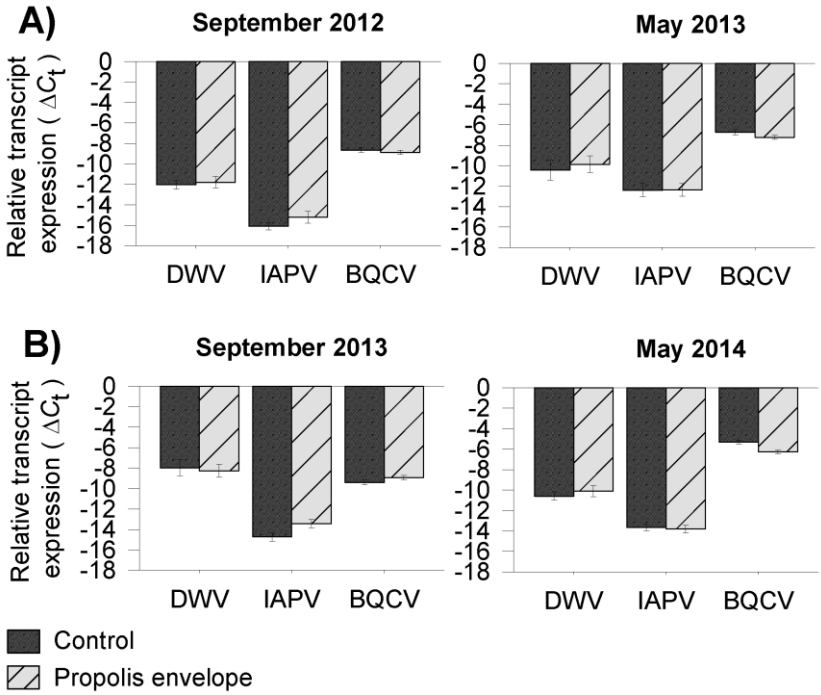

Figure 2.5. Relative expression levels of DWV, IAPV and BQCV normalized to the reference genes actin and RPS-5. Gene transcript average ± SEM (N = 20 biological replicates per colony and 6 colonies per treatment) for A) September 2012 and May 2013, and B) September 2013 and May 2014 for controls and propolis envelope treatment colonies. Negative levels indicate viral load expression was lower than reference gene expression.

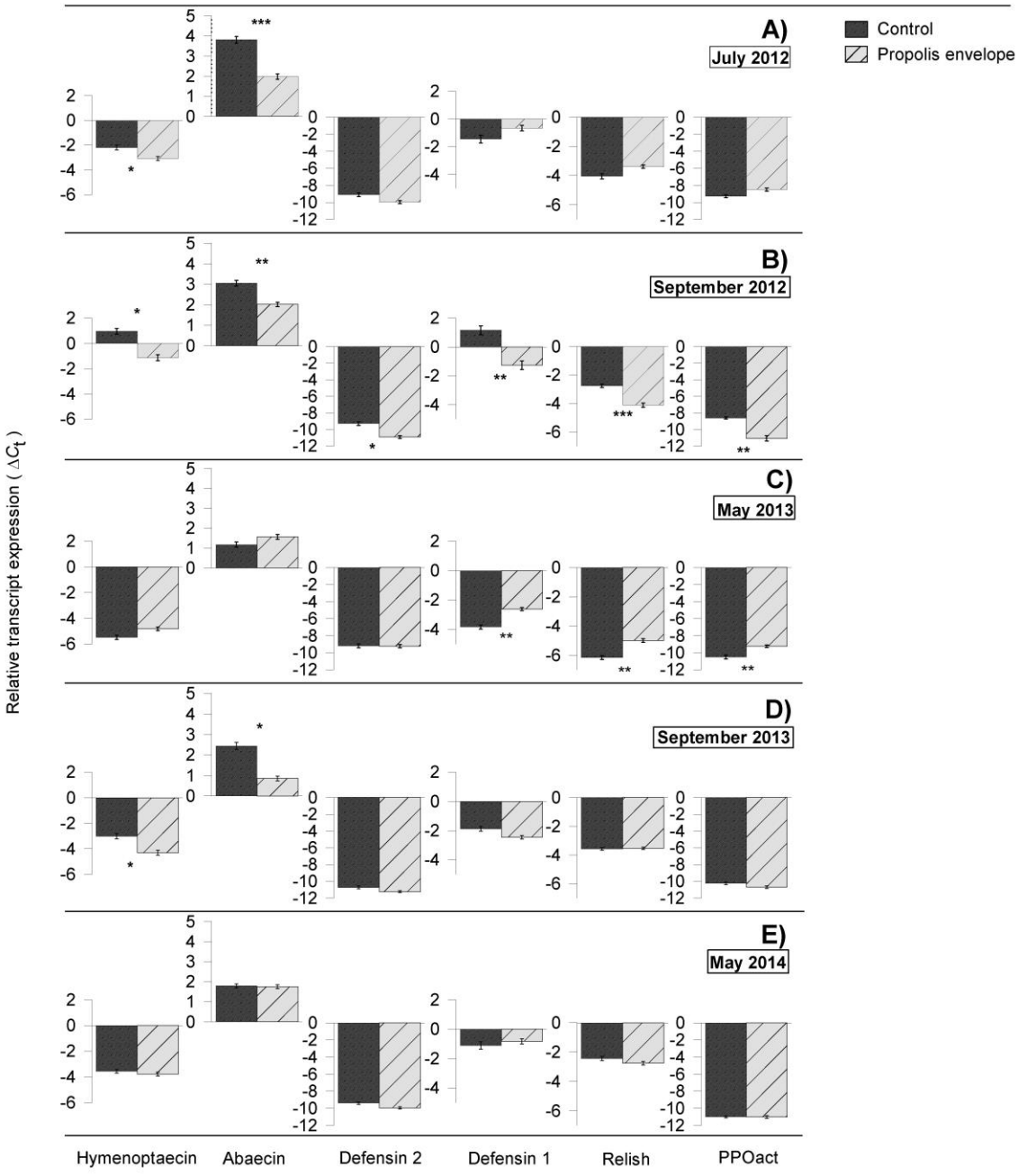

Figure 2.6. Relative expression levels of hymenoptaecin, abaecin, defensin-2, defensin-1, relish and phenoloxidase normalized to the reference genes actin and RPS-5. Gene transcript average ± SEM (N = 20 biological replicates per colony and 6 colonies per treatment) for A) July 2012, B) September 2012, C) May 2013, D) September 2013 and E) May 2014 for controls and propolis envelope treatment colonies.

Significant difference between groups, determined by two tailed t-test with colonies as random variables, indicated by * = P < 0.05; ** = P < 0.01; *** = P < 0.001). A low value indicates lower gene expression (e.g. in July 2012, colonies with a propolis envelope had significantly lower expression of both hymenoptaecin and abaecin relative to controls).

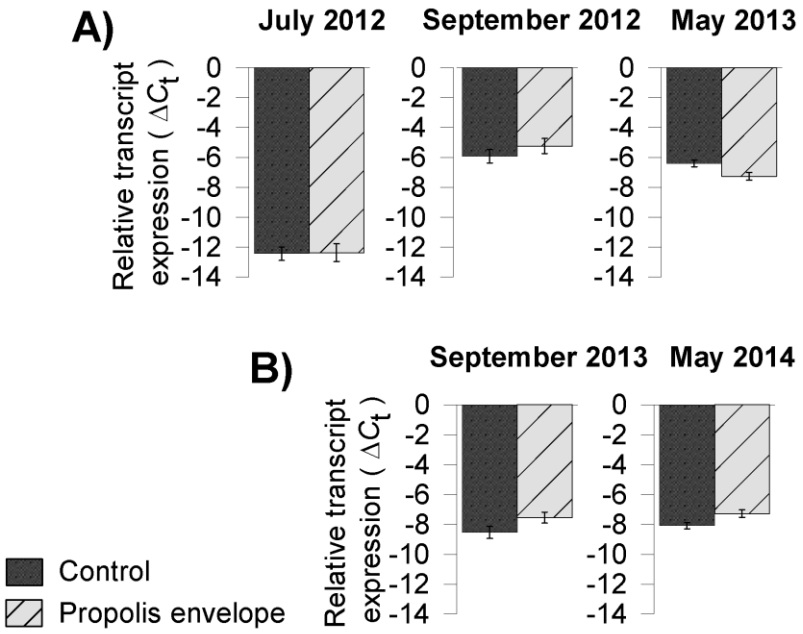

Figure 2.7. Relative expression levels of eubacterial 16S gene normalized to the reference genes actin and RPS-5. Gene transcript average ± SEM (N = 20 biological replicates per colony and 6 colonies per treatment) for A) July 2012, September 2013, May 2013, and B) September 2013 and May 2014 for controls and propolis envelope treatment colonies.

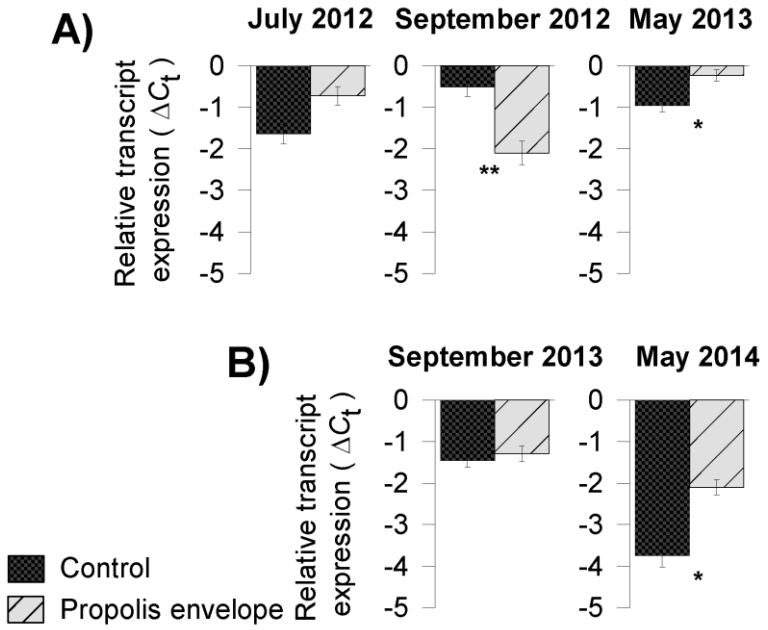

Figure 2.8. Relative expression levels of vitellogenin normalized to the reference genes actin and RPS-5. Gene transcript average ± SEM (N = 20 biological replicates per colony and 6 colonies per treatment) for A) July 2012, September 2013, May, and B) September 2013 and May 2014 for controls and propolis envelope treatment colonies. Significant difference between groups, determined by two tailed t-test with colonies as random variables, indicated by * = $P < 0.05$; ** = $P < 0.01$.

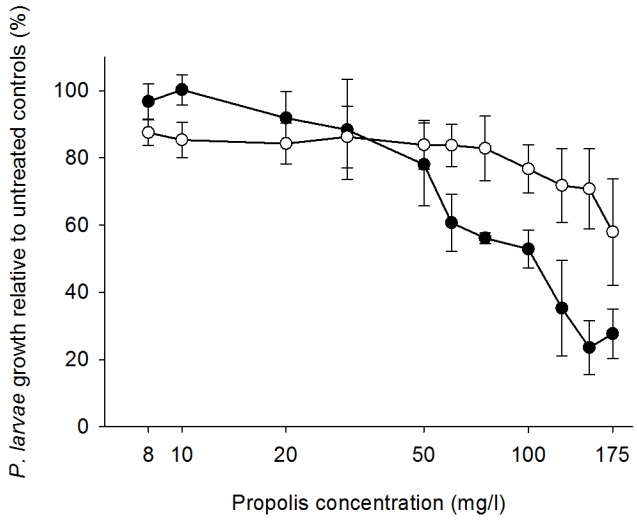

Figure 2.9. Dose responsiveness of *P. larvae* growth to propolis extracts collected in October (black circles) and April (white circles). *P. larvae* growth was measured as a percent optical density (OD_{600}) relative to untreated controls.

2.8 Tables

Locus	Gene ID	Category	Forward Primer	Reverse Primer	Reference
VGMC(vitellogenin)	UGID:1213462	Development	AGTTCCGACCGACGACGA	TTCCCTCCCACGGAGTCC	Boncristiani et al., 2012
actin	GB17681	House keeping	TTGTATGCCAACACTGTCCTTT	TGGCGCGATGATCTTAATTT	Boncristiani et al., 2012
RPS5	GB11132	House keeping	AATTATTTGGTCGCTGGAATTG	TAACGTCCAGCAGAATGTGGTA	Evans et al., 2006
abaecin	GB18323	Immune	CAGCATTCGCATACGTACCA	GACCAGGAAACGTTGGAAAC	Evans et al., 2006
defensin2	GB10036	Immune	GCAACTACCGCCTTTACGTC	GGGTAACGTGCGACGTTTTA	Evans et al., 2006
defensin1	GB19392	Immune	GGATGAATTCGAGCCACTTG	ATGACCTCCAGCTTTACCCA	Designed using MacVector
hymenopt	GB17538	Immune	CTCTTCTGTGCCGTTGCATA	GCGTCTCCTGTCATTCCATT	Evans et al., 2006
PPOact	GB18767	Immune	ATCCAACAGAGTGGCCTTGG	GAAATCGTATTCGCCGAGC	Designed using MacVector
relish	GB13742	Immune	AGCAGTGTTGAAGGAGCTGA	AAGCGTCCATAATCACACCA	Designed using MacVector
Bact16S (774/1391)	M60313	Pathogen	GTAGTCCACGCTGTAAACGATG	GACGGGCGGTGTGTRCA	Simone et al., 2009
BQCV	HQ655494.1	Pathogen	TTTAGAGCGAATTCGGAAACA	GGCGTACCGATAAAGATGGA	Boncristiani et al., 2012
DWV	AY292384.1	Pathogen	GAGATTGAAGCGCATGAACA	TGAATTCAGTGTCGCCCATA	Boncristiani et al., 2012
IAPVF1aR1	EF219380.1	Pathogen	GCGGAGAATATAAGGCTCAG	CTTGCAAGATAAGAAAGGGGG	Boncristiani et al., 2012

Table 2.1. Locus (common) names, Gene identification numbers, gene category (development, immune, house keeping), primers sequences and references (when selected from the literature) for genes tested via real time PCR.

A) 2012-2013	Absolute deviation from the group median		Levene test
	Control	Propolis envelope	
Hymenoptaecin	2.86	1.90	$F_{1,729} = 56.3313$ $P < 0.0001$
Abaecin	1.54	1.10	$F_{1,723} = 35.4422$ $P < 0.0001$
Defensin-2	1.96	1.72	$F_{1,733} = 5.7569$ $P = 0.02$
Defensin-1	2.30	1.55	$F_{1,368} = 26.8223$ $P < 0.0001$
Relish	1.54	0.98	$F_{1,372} = 41.7818$ $P < 0.0001$
Phenoloxidase	1.10	1.34	$F_{1,322} = 4.9739$ $P = 0.03$
B) 2013-2014	Absolute deviation from the group median		Levene test
	Control	Propolis envelope	
Hymenoptaecin	1.67	1.28	$F_{1,457} = 12.7419$ $P = 0.0004$
Abaecin	1.22	0.89	$F_{1,454} = 17.5586$ $P < 0.0001$
Defensin-2	1.43	1.12	$F_{1,459} = 11.1749$ $P = 0.0009$
Defensin-1	1.42	1.15	$F_{1,263} = 5.0091$ $P = 0.0261$
Relish	0.83	0.70	$F_{1,267} = 3.5282$ $P = 0.0614$
Phenoloxidase	1.14	0.88	$F_{1,240} = 6.1731$ $P = 0.0137$

Table 2.2. Variability of the combined immune gene transcription data for each experimental year, A) 2012-2013 and B) 2013-2014. The spread of the immune gene expression data is measured as $zij = |yij - \bar{y}i|$ and reported as the absolute deviation of each immune response (ΔC_t) and the group median, for each gene separately.

	September		Statistical analysis
	2012	2013	
Varroa mite (*Varroa* mites/100 bees)	4.96 ± 1.48	9.91 ± 2.96	$Z = 2.22$ $P = 0.03$
DWV (relative transcript expression)	- 11.93 ± 0.35	- 8.09 ± 0.54	$F_{1,25} = 9.12$ $P = 0.006$
	May		Statistical analysis
	2013	2014	
Varroa mite (*Varroa* mites/100 bees)	0.79 ± 0.92	2.57 ± 2.20	$Z = 2.02$ $P = 0.04$
Nosema (10^5) (*Nosema* spp. spores/100 bees)	3 ± 0.55	6.1 ± 1.62	$Z = 2.52$ $P = 0.02$
BQCV (relative transcript expression)	- 6.96 ± 0.16	- 5.68 ±0.13	$F_{1,23} = 8.25$ $P = 0.009$

Table 2.3. Average levels (± SEM) of natural occurring pathogens (virus and *Nosema*) and parasites (*Varroa* mite) between years for September and May. Gene expression analysis of DWV and BQCV was performed using ANOVA, using R version 2.15, with colony as a random factor and year as a fixed effect. Colony-level measurements (*Varroa* and *Nosema* levels) were compared between years using two tailed t-tests and non-parametric Kruskal-Wallis tests, as appropriate, using R version 2.15.

Chapter 3.

Propolis envelope in *Apis mellifera* colonies induces honey bees' antimicrobial defenses against the pathogen, *Paenibacillus larvae*

Summary

Honey bees have immune defenses as individuals and as a colony (e.g., individual and social immunity). Social immunity describes colony level anti-parasitic and anti-pathogenic protection, and one form of social immunity in honey bees is the collection and deposition of antimicrobial plant resins in the nest, called propolis. In this study, we tested the effects of the propolis envelope as a natural defense against *Paenibacillus larvae*, the causative agent of American foulbrood (AFB) disease, on the expression of antimicrobial peptides in 7-d old nurse bees, the antimicrobial activity of larval food, and on the clinical signs of American foulbrood within the colony. The immune system activity of nurse bees was measured via real-time PCR, using primers for three honey bee antimicrobial peptides (hymenoptaecin, apidaecin and defensin-1). A bacterial growth assay was performed to assess the inhibitory activity of larval food from 1-2 day old larvae, the only susceptible stage to AFB, against the growth of *P. larvae*. Our results show that both nurse bee immune system activity and the antimicrobial activity of larval food, were significantly higher when challenged colonies had a propolis envelope compared to when they did not have the envelope. In addition, colonies with a propolis envelope had significantly reduced levels of American foulbrood clinical signs two months following challenge. The propolis envelope serves as an external antimicrobial layer around the colony, providing a therapeutic defense against infectious pathogens and increasing the protective physiological response of nurse bees towards bee brood.

3.1 Introduction

The nests of densely populated social insect colonies provide a favorable habitat for a wide range of parasites and pathogens (Schmid-Hempel, 2005) that have evolved to overwhelm or suppress their hosts' immune defenses. In turn, insect societies have evolved remarkable abilities to counter these challenges via dynamic defense mechanisms at both the individual level (individual immunity; Evans et al., 2006) and the colony level (social immunity; Cremer et al., 2007).

An example of social immunity in honey bees, *Apis mellifera*, is the collection of antimicrobial plant resins and the deposition of the resins on the interior walls of the nest, where it is called a propolis envelope (Seeley and Morse, 1976; Simone-Finstrom and Spivak, 2010). Previous work has shown that the antimicrobial activity of the propolis envelope provides constitutive benefits to adult bees' immunity. In an apparently healthy colony, an experimentally applied propolis envelope (ethanol extract of propolis, painted inside the hive box) appeared to lower general bacterial loads (as measured by 16S rRNA) within the colony, resulting in a decreased need of bees to activate the immune system to fight off microbes (Simone et al., 2009). In another experiment, when bees in apparently healthy colonies built a natural propolis envelope, there was no difference in general bacterial loads between colonies with or without a propolis envelope, but the baseline expression of immune-related genes (immune system activation) of individual bees was significantly lower over the entire foraging season in colonies with a propolis envelope (Chapter 2). Similar results have been observed in social wood ants (*Formica paralugubris*), a species that constitutively collects plant resins and places globules of resin near the brood (Brütsch and Chapuisat, 2014), resulting in reduced growth of microorganisms (Christe et al., 2003), and lower immune system activity of adult worker ants (Castella et al., 2008). The immune system is the most costly physiological system in insects (Evans and Pettis, 2005; Schmid-Hempel, 2005); thus a reduction in its activation, especially over time, result in great fitness benefits to the individual and colony (Chapuisat et al., 2007; Chapter 2).

In addition to the constitutive benefits of the propolis envelope to the bees' immune system, the antimicrobial properties of propolis may promote an inducible and

therapeutic defense against pathogens. Simone-Finstrom and Spivak (2012) demonstrated the first case of self-medication in honey bees. Colonies experimentally challenged with *Ascosphaera apis* increased resin collection in response to the brood fungal pathogen compared to unchallenged colonies (Simone-Finstrom and Spivak, 2012). Moreover, other colonies with an experimentally applied propolis envelope had significantly lower clinical signs of this fungal disease compared to colonies with no envelope (Simone-Finstrom and Spivak, 2012). *In vitro* studies have demonstrated the inhibitory activity of propolis, and specific compounds within propolis, to the growth of a highly infectious bacterial pathogen of honey bees, *Paenibacillus larvae,* the causative agent of American foulbrood disease, and to *A. apis*, the fungal agent of chalkbrood disease (Bastos et al., 2008; Bilikova et al., 2013; Lindenfelser, 1968; Wilson et al., 2013; Wilson et al., 2015). However, the mode of action by which the propolis envelope induces and enhances colony defense mechanisms against pathogens is largely unknown.

Here we studied if a natural propolis envelope in a honey bee hive helps promote a therapeutic defense at the individual and colony level after challenge with *P. larvae*. Young bee larvae (1-2 d old), the only susceptible life-stage to this pathogen, become infected with *P. larvae* spores via oral intake of contaminated larval food (Shimanuki, 1990). Young larvae are thought to have lower immunological defenses compared to adults (lower hemolymph cell counts and phenoloxidase activity; Chan et al., 2009; Wilson-Rich et al., 2008), and therefore may rely mostly on social immunity to help fight brood diseases. For example, some colonies display hygienic behavior, whereby the adult bees detect and quickly remove American foulbrood (AFB) infected brood from the nest before the pathogen becomes infectious (Rothenbuhler, 1964; Spivak and Reuter, 2001). Additionally, nurse bees secrete antimicrobial compounds into larval food, which protect the larvae from *P. larvae* infection (Rose and Briggs, 1969; Thompson and Rothenbuhler, 1957). Two other documented mechanisms of colony resistance to AFB include the removal of *P. larvae* spores from contaminated honey by action of the honey stopper (Sturtevant and Revell, 1953) and the genetic ability of larvae to resist AFB infection (Evans, 2004; Thompson and Rothenbuhler, 1957).

We investigated the effects of the propolis envelope as a natural defense against *P. larvae* on the antimicrobial activity of larval food, the expression of antimicrobial peptides in 7-d old nurse bees, and the overall reduction of clinical signs of AFB by the colony. We hypothesized that: 1) larval food antimicrobial activity and 2) immune-related gene expression in 7-d old bees would be higher in colonies challenged with *P. larvae*, especially in the presence of a propolis envelope (Chan et al., 2009; Evans, 2004; Evans and Pettis, 2005; Rose and Briggs, 1969), and 3) clinical signs of AFB would be reduced in *P. larvae* challenged bee colonies with a propolis envelope compared to challenged colonies without the envelope (Simone-Finstrom and Spivak, 2012). Our findings showed that 10 days after challenge, the antimicrobial activity of larval food was significantly higher in challenged colonies with a propolis envelope compared to its activity in challenged colonies with no propolis envelope. Bees from challenged colonies with a propolis envelope invested more in the production of antimicrobial peptides compared to bees in challenged colonies without a propolis envelope, suggesting that the source of the antimicrobial compounds in larval food may be, at least in part, from antimicrobial peptides. Finally, our results revealed significant reduction in the clinical signs of AFB infection in colonies with a propolis envelope over time. This study emphasizes the critical importance of the propolis envelope to honey bees' health and demonstrates its therapeutic role to both larvae and adults. The propolis envelope can be viewed as an external component of the bees' immune system and thus as a vital part of honey bee colony defense.

3.2 Materials and Methods

3.2.1 Experimental design

Twenty, 4-frame "nucleus" colonies were purchased from a commercial beekeeper in May 2013 and established in 10-frame equipment at the University of Minnesota, Saint Paul campus. Hygienic tests (Büchler et al., 2013) were performed on all colonies to

ensure the colony did not display hygienic behavior, a behavioral mechanism of resistance to American foulbrood in which the bees detect and remove immature bees infected with *P. larvae* (Spivak and Reuter, 2001). A 2x2 factorial design was employed in this study. Ten colonies were provided with commercially available propolis traps stapled to the four inner walls of each bee box to encourage the bees to construct a propolis envelope within the nest, following previous methods (Chapter 2). Colonies that were provided with propolis traps deposited propolis in most (over 80%) of the slits in the traps, creating a propolis envelope (Figure 3.1). Five of the ten colonies with a propolis envelope were challenged with *P. larvae* (propolis + *P. larvae* treatment) and the other five were left unchallenged (propolis + no *P. larvae* treatment). The remaining ten colonies were not provided with propolis traps and the bees deposited propolis in the cracks and crevices within the box where they could. Five of the ten colonies without a propolis envelope were challenged with *P. larvae* (no propolis + *P. larvae* treatment) and the other five were left unchallenged (no propolis + no *P. larvae* treatment).

3.2.2 Colony inoculation with *P. larvae*

Sugar solution containing 10^7 *P. larvae* spores/ml was prepared by removing 100 AFB infected scales from diseased colonies, macerating and suspending the crushed scales in sucrose-water (1:10 w/v) (de Graaf et al., 2013). The concentration was confirmed using a haemocytometer. Colonies were challenged with *P. larvae* on July 31st 2013 by spraying 5 ml of the spore sugar solution on each comb within the colony (Seeley and Tarpy, 2007). Unchallenged colonies were sprayed with 5 ml of sugar solution (1:10 w/v) on each comb within the colony.

3.2.3 Larval food collection

Larval food from 1-2 day old larvae was collected 9 days after colony inoculation with *P. larvae* (asymptomatic period, August 9th) and after the presence of clinical signs (symptomatic period, September 12th, 43 days after challenge). Prior to larval food

collection, an empty frame was introduced into the colony and marked when eggs were present. Three days after the frames were marked, when 1-2 day old larvae were present, the frames were removed and larval food was collected following Schmitzová et al. (1998). In a temperature-controlled room, each young larva was removed from the cell using a sterile grafting tool, the larval food from each cell was individually homogenized in 30 µl of phosphate buffer by repeated pipetting and then transferred to a 1.5 ml Eppendorf tube. Larval food from 32 cells, located in the same frame, was collected from each colony and stored individually.

3.2.4 Larval food antimicrobial assay

A bacterial growth assay was performed to assess the inhibitory activity of larval food from 1-2 day old larvae against the growth of *P. larvae*. Phosphate buffer was removed from each sample by freeze-drying the larval food and controls (30 µl of phosphate buffer from each sample). *P. larvae* (from stock strains obtained from the USDA Agricultural Research Service culture collection; NRRL# B-2605) were cultured in brain/heart infusion broth and the bacterial growth assay was conducted following Wilson et al. (2015). Dried larval food samples were resolubilized in 100 µL of brain/heart infusion broth, transferred to 96 well plates and placed in a plate shaker for 30 min at 400 rpm to improve homogenization. *P. larvae* liquid culture was transferred into the well plates (creating a 1:100 dilution of *P. larvae* in each well), and the well plates were incubated at 37°C at 400 rpm for six hours. Bacterial growth inhibition was evaluated in 96-well plates by measuring turbidity (optical density at time 0h subtracted from time 6h, OD_{600}) of treated cultures (containing larval food) relative to untreated controls (phosphate buffer only) using a microplate spectrophotometer.

3.2.5 Seven day old bees sample collection for gene expression analysis

Nine days after colony inoculation with *P. larvae* (asymptomatic period; August 9th) and after the presence of clinical signs (symptomatic period; September 12th, 43 days

after challenge), 25 7-day old bees were collected from every colony. Newly emerged bees (noted by their location near eclosing adults from pupal cells, and by their fuzzy appearance; Human et al., 2013) were painted using enamel paint markers and collected after six days. The marked, 7-d old bees were stored in -80 ˚C freezer until analysis. Immunocompetence in bees increases from emergence to day 7-8 of adult life, at which time it is thought that their immune system is fully capable of starting an immune response (Wilson-Rich et al., 2008). We sampled 7-d old bees because immune activity becomes more variable after eight days until bees become foragers, when immunity is highly decreased (Amdam et al., 2005; Evans et al., 2006).

Gene candidates for the immune response included the antimicrobial peptides (AMPs): hymenoptaecin, apidaecin and defensin-1. We also estimated the effects of the propolis envelope on levels of general bacteria which would include *P. larvae*, by measuring the gene expression of 16S rRNA of eubacteria in individual bees using a universal primer pair for the eubacterial 16S gene (Evans, 2006;Weisburg et al., 1991).

3.2.6 RNA extraction, cDNA synthesis and real-time PCR analysis

Total RNA was isolated from individual abdomens of 7-d old marked bees using TRIzol reagent (Ambion, Austin, TX) following the manufacture's protocol. Quality and quantity of total RNA was measured using NanoDrop2000 instrument (Thermo Scientific Inc.) and 3.5 µg of each sample was used for cDNA synthesis. Prior to cDNA synthesis, RNA was treated with DNAse I (Ambion) and reverse transcription for cDNA synthesis was carried out using Superscript II (Invitrogen). Complementary DNA was diluted 1:3 with RNAse and DNAse free water. Relative quantification of candidate genes used to assess the immune system response and bacterial levels were analyzed via real-time PCR (Biorad CFX96). Samples for real-time PCR were prepared using iTaq Universal SYBR Green Supermix (Biorad). Primer sequences used for this experiment were selected from the literature when available and optimal annealing temperature was met (Table 3.1). Otherwise, primers were designed using MacVector version 12.5.1 and specificity was confirmed using primerBLAST.

3.2.7 Level of American foulbrood infection assessment

The number of larvae with clinical signs of AFB (sunken wax capping and uncapped cells containing discolored, ropy brood) was quantified approximately every 15 days after the appearance of the first clinical signs (August 30th, September 16th and October 1st). A severity score from 0-3 was given for each comb that contained larvae: 0 = 0 cells containing signs of AFB; 1 = 1-5 cells; 2 = 6-25 cells; and 3 = ≥ 26 cells per comb (Spivak and Reuter, 2001).

3.2.8 Statistical analysis

The inhibitory activity of larval food to *P. larvae* growth was analyzed by ANOVA with treatment as a fixed effect using R version 2.15, as there was no interaction between main factors. *P. larvae* growth was calculated by subtracting the optical density at time 0h from time 6h (OD_{600}). The percentage of *P. larvae* growth in the presence of larval food relative to untreated controls (phosphate buffer only) was pooled by colony and compared among treatments. Larval food antimicrobial activity from unchallenged colonies did not vary between the two collection periods and therefore, correction for temporal variation was not needed.

Gene expression analysis of antimicrobial peptides was conducted using the $\Delta\Delta C_t$ method to correct for temporal variation in gene expression between the two collection periods (Livak and Schmittgen, 2001). C_t values for all four treatment groups were first normalized to two reference genes (actin and RPS-5) and second, normalized C_t values of bees from challenged colonies were normalized to their respective controls. Unchallenged colonies with and without a propolis envelope served as controls for the respective challenged colonies with and without a propolis envelope (e.g., propolis + no *P. larvae* treatment colonies served as controls for propolis + *P. larvae* treatment colonies). Finally, the normalized expression between the propolis + *P. larvae* and no

propolis + *P. larvae* treatments was compared using two tailed t-test, using R version 2.15, with colony of origin as a random factor and treatment group as a fixed effect.

Clinical signs of American foulbrood in colonies from August, September and October were compared between treatment groups (propolis + *P. larvae* and no propolis + *P. larvae* treatments; unchallenged colonies did not show AFB clinical signs) using two tailed t-tests with colony of origin as a random factor, using R version 2.15.

3.3 Results

3.3.1 Larval food antimicrobial activity

Larval food from 1-2 day old larvae from colonies with and without a propolis envelope showed differences in the ability to inhibit the growth of *P. larvae* both before and after the appearance of AFB clinical signs in the colonies.

Asymptomatic period, August: Larval food collected from challenged colonies with a propolis envelope showed significantly higher inhibition of *P. larvae* growth (lower growth relative to controls) compared to larval food from challenged and unchallenged colonies without a propolis envelope ($F_{3,16} = 4.26$, $P = 0.02$; Figure 3.1a). In colonies without a propolis envelope, there was no difference in larval food bioactivity whether the colonies were challenged or unchallenged

By September, when challenged colonies had clinical signs of AFB, larval food collected from challenged colonies with a propolis envelope continued to show significantly higher inhibitory activity against *P. larvae* but only compared to larval food from unchallenged colonies without a propolis envelope ($F_{3,16} = 4.79$, $P = 0.01$; Figure 3.1b). Larval food from unchallenged colonies with a propolis envelope and challenged colonies without a propolis envelope had intermediate levels of inhibition.

3.3.2 Immune-related gene expression

Asymptomatic period; August: Gene expression analysis of three AMPs (hymenoptaecin, apidaecin and defensin-1) in 7-d old bees collected before the appearance of AFB clinical signs (9 days after the challenge with *P. larvae*) had slightly higher, but not significantly different, expression of hymenoptaecin and apidaecin in bees from challenged colonies with a propolis envelope compared to challenged colonies without a propolis envelope. In contrast to the other two AMPs, gene expression of defensin-1 in bees from challenged colonies without a propolis envelope was marginally higher compared to bees in challenged colonies with a propolis envelope ($F_{1,8} = 4.96$, $P = 0.057$; Figure 3.2a).

Symptomatic period; September: After the appearance of AFB clinical signs (43 days after the challenge), bees from challenged colonies with a propolis envelope had significantly higher transcription of hymenoptaecin ($F_{1,8} = 5.84$, $P = 0.035$) and apidaecin ($F_{1,8} = 7.67$, $P = 0.031$) compared to challenged colonies without a propolis envelope. The gene transcription of defensin-1 in bees from challenged colonies without a propolis envelope changed from being slightly up-regulated (as observed in August) to being similar in transcription levels to bees from challenged colonies with a propolis envelope (Figure 3.2b).

3.3.3 Level of American foulbrood infection

Colonies first showed clinical signs of AFB on August 30th, 30 days after challenge. Colonies with a propolis envelope that were challenged with *P. larvae* had slightly fewer larvae with clinical signs of AFB on August 30th and September 16th compared to challenged colonies without a propolis envelope, but this difference was not significantly different until October 1^{st,} when colonies with a propolis envelope had significantly fewer larvae with clinical signs of AFB ($F_{1,8} = 7.98$, $P = 0.022$; Figure 3.5). The presence of the propolis envelope did not completely clear AFB infection; all colonies had clinical signs by the end of the experiment. However the severity of clinical signs in October in colonies with a propolis envelope was relatively mild (score just over 1, or 1-5 infected

larvae per comb) compared to the severity in colonies without the propolis envelope (score just over 2, or 6-25 infected larvae per comb).

3.4 Discussion

The presence of a propolis envelope in managed honey bee colonies provides quantifiable benefits to honey bee immunity (Simone et al., 2009; Chapter 2). Propolis has a constitutive benefit on the immune system of adult bees by lowering the baseline expression of immune-related genes resulting in a decreased need to activate the immune system (Simone et al., 2009; Chapter 2). In this study, we demonstrate the benefits of the propolis envelope to bees' immune system after field colonies were challenged with a highly infectious bacterial pathogen, *P. larvae*, the causative agent of American foulbrood disease. The presence of a propolis envelope improved individual and colony defense mechanisms against *P. larvae* via three pathways. First, before the colonies were symptomatic, the inhibitory activity of larval food against *P. larvae* was significantly higher in challenged colonies with a propolis envelope compared to challenged colonies without a propolis envelope. Second, the presence of a propolis envelope improved the capacity of nurse age bees to mount a stronger immune response (7-day old bees had significantly higher gene expression levels of two antimicrobial peptides, hymenoptaecin and apidaecin) in the presence of AFB signs, compared to bees in colonies with no propolis envelope. Lastly, the presence of a propolis envelope inside a colony significantly reduced the number of larvae with clinical signs of AFB two months after *P. larvae* challenge. Our findings add to those of Simone-Finstrom and Spivak (2012) who showed that a propolis envelope reduced the clinical signs of a different brood disease, chalkbrood, after experimental challenge with the fungal pathogen *A. apis*.

3.4.1 Larval food antimicrobial activity

In August, before the colonies were symptomatic, the larval food in challenged colonies with a propolis envelope significantly reduced the growth of *P. larvae in vitro* compared to the larval food in both challenged and unchallenged colonies without a propolis envelope. Interestingly, the larval food in unchallenged colonies with a propolis envelope had intermediate levels of antibacterial activity, suggesting that compounds from the propolis envelope play a role in the bioactivity of larval food against bee pathogens. Although the propolis envelope may not come into direct contact with larval food, volatile compounds present in propolis can diffuse through the hive, and may contribute to the complex way in which bees fight infections. Several studies have confirmed the activity of propolis volatiles against Gram-positive (Bankova et al., 1998; Bastos et al., 2008; Kujumgiev et al., 1999; Melliou et al., 2007; Oliveira et al., 2010), and Gram-negative bacteria (Melliou et al., 2007; Oliveira et al., 2010; Simionatto et al., 2012). However, it has never been investigated if propolis compounds are present in larval food, although it has been suggested that antimicrobial compounds found in propolis are present in honey (Mao et al., 2013).

The presence of *P. larvae* or a propolis envelope alone did not contribute to higher antimicrobial activity of larval food in both asymptomatic and symptomatic periods. It was only when colonies had both factors (propolis envelope and *P. larvae* challenge) that the larval food bioactivity was significantly higher than unchallenged colonies without a propolis envelope. One of the earliest studied mechanisms of AFB resistance was associated with larval food. Thompson and Rothenbuhler (1957) suggested that nurse bees from AFB resistant-lines secrete anti-foulbrood compounds into larval food as a defense mechanism against *P. larvae*. It was later confirmed that larval food from AFB resistant-lines was, indeed, more effective at reducing *P. larvae* growth than larval food from susceptible colonies (Rose and Briggs, 1969). Our results support previous studies (Rose and Briggs, 1969; Thompson and Rothenbuhler, 1957), and confirm the existence of a natural defense mechanism in honey bees against AFB when nurse bees feed larvae glandular food with a higher antimicrobial activity. However, we observed that this mechanism of defense against AFB was most effective only when colonies had a propolis envelope. A synergism between propolis and antimicrobial, as well as antifungal,

substances have been previously reported to augment the effect of commonly used drugs to combat human diseases (Orsi et al., 2012; Pippi et al., 2015). The relatively high bioactivity of the larval food from challenged colonies with a propolis envelope suggests there may be a synergistic effect between propolis and antimicrobial substances present, or incorporated, into larva food.

Previous research investigating the antagonistic effect of lactic acid bacteria (LAB) from honey bee stomach on *P. larvae*, demonstrated its efficacy in reducing the number of AFB-infected larvae when LAB was added to larval food (Forsgren et al., 2010). Interestingly, midgut contents of 8-d old nurse bees have higher inhibitory activity on the growth of *P. larvae* compared to 1-d old bees and foragers (Crailsheim and Riessberger-Gallé, 2001). Carpenter ants fight bacterial infection in a slightly different way. A proposed mechanism underlying bacterial defense in carpenter ants (*Camponotus pennsylvanicus*) is through increased trophallaxis between infected workers and naïve nestmates, which was considered as an example of social immunization (Hamilton et al., 2011). During trophallaxis, infected workers share droplets of antimicrobial peptides with naïve individuals, improving the survival of naïve individuals upon future contact with the same pathogen. The consistently higher antimicrobial activity of larval food in challenged colonies with propolis reveals a therapeutic effect of the propolis envelope and a protective physiological response of nurse bees towards the brood. The source of the growth-inhibiting substances put in larval food, from glandular secretions (e.g. antimicrobial peptides; Bilikova et al., 2001; Hamilton et al., 2011) or LAB symbionts (e.g. Forsgren et al., 2010), requires further study.

3.4.2 Immune-related gene expression

In previous work, the presence of a propolis envelope within the nest reduced bees' need to activate the immune system, as measured by lower immune-related gene expression in 7-d old bees in unchallenged and apparently healthy colonies (Simone et al., 2009; Chapter 2). Here we show an additional effect of the propolis envelope: under pathogenic stress, bees in challenged colonies with a propolis envelope have the ability to

mount a stronger immune response compared to bees in challenged colonies without a propolis envelope.

Gene expression analysis of nurse age bees collected 43 days after *P. larvae* challenge, when the colony was symptomatic, showed significantly higher transcription of hymenoptaecin and apidaecin in colonies with a propolis envelope compared to bees in challenged colonies without a propolis envelope. The increase in transcription of these AMP's were evident during the asymptomatic phase, but was not statistically different during that time. It is well known that honey bees increase the expression of most antimicrobial peptides, including hymenoptaecin and apidaecin, to fight a *P. larvae* infection (Chan et al., 2009; Evans, 2006; Evans et al., 2006). The higher expression of hymenoptaecin and apidaecin in bees from challenged colonies with a propolis envelope demonstrates their ability to synthesize higher levels of antimicrobial compounds and potentially decrease colony-level AFB infection more rapidly and efficiently compared to bees from challenged colonies without a propolis envelope. These findings support the hypothesis that nurse age bees incorporate antimicrobial substances into larval food, and suggests that the source of these substances are, at least in part, from antimicrobial peptides. These findings also demonstrate that the lower immune system activation of bees in apparently healthy colonies with a propolis envelope is not due to immune suppression (Simone et al., 2009; Chapter 2); as bees in *P. larvae* challenged colonies with a propolis envelope were able to mount a strong immune response.

Gene transcription of defensin-1 was marginally lower in bees from challenged colonies with a propolis envelope during the asymptomatic period. After clinical signs of AFB were present, both treatment groups had very low expression levels of defensin-1. The low expression of defensin-1 after *P. larvae* challenge observed in this study is supported by a number of other studies (Casteels-Josson et al., 1994; Chan et al., 2009; Evans, 2004; Evans and Pettis, 2005) who also found that its expression was minimal after bacterial challenge.

3.4.3 American foulbrood infection level

American foulbrood is a highly infectious disease that spreads easily within the colony as each infected larva produces an average of 2.5 x 10^9 spores (Ratnieks, 1992). *P. larvae* are endospore-forming bacteria; after perforating the midgut cell wall, less than twenty endospores can effectively cause mortality of 1-d old bee larvae. Our results indicate that the presence of a propolis envelope inside a colony significantly reduced the number of larvae with clinical signs of AFB over time, but did not eliminate the disease completely. Colonies challenged with *P. larvae*, with and without propolis envelopes, had similar levels of AFB infection in late August and mid-September. By early October when queens were still actively laying eggs, infection levels in colonies with a propolis envelope were significantly lower compared to levels in colonies without a propolis envelope.

Previous studies have demonstrated four different mechanisms of colony resistance to AFB: 1) removal of *P. larvae* spores from contaminated honey by action of the honey stopper (Sturtevant and Revell, 1953); 2) detection and removal of infected larvae by adult bees before *P. larvae* produces infectious spores (hygienic behavior; Spivak and Reuter, 2001); 3) genetic ability of larvae to resist AFB infection (Evans, 2004; Thompson and Rothenbuhler, 1957), and 4) high inhibitory activity of larval food on the growth of *P. larvae* (Rose and Briggs, 1969). In our study, we demonstrate a fifth mechanism of colony resistance: the presence of a propolis envelope in the colony, which increases the individual (production of AMPs) and collective (increased bioactivity of larval food) immune responses of bees. We suggest that the reduced level of AFB clinical signs in early October in colonies with a propolis envelope compared to colonies without a propolis envelope is a result of a combination of the effects of propolis on both the collective and individual behavioral responses (larval food bioactivity and individual bee immune response).

3.5 Conclusion

Pathogens evolve to overwhelm their host's immune defenses while hosts evolve mechanisms of resistance to overcome pathogen infection. The process of domestication of *Apis mellifera* by keeping them in man-made hives has interfered with a critical, natural defense mechanism of the honey bee colony: the bees do not construct a natural propolis envelope inside the hive as they do in natural tree cavities. Our results strongly indicate that the propolis envelope serves as an external antimicrobial layer around the colony, protecting the brood from *P. larvae* infection and supporting bees' ability to induce a strong and effective immune response resulting in a lower infection load two months following the challenge.

3.6 Acknowledgments

We would like to thank Gary Reuter and Christine Kulhanek (University of Minnesota) for assistance with honey bees' colony management, and Dr. Michael Goblirsch (University of Minnesota) for valuable discussions. We also acknowledge the support of all the members of the Bee Lab at University of Minnesota.

3.7 Figures

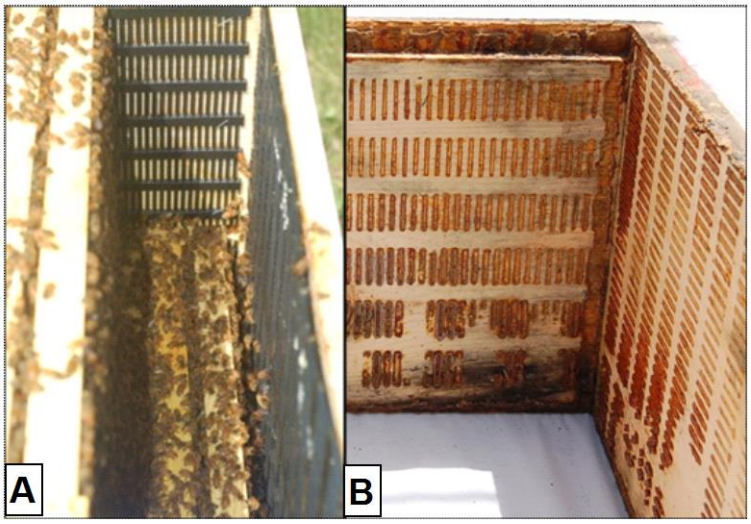

Figure 3.1. Propolis envelope treatment bee box. A) Propolis traps stapled to inside walls of a hive to encourage bees to construct a propolis envelope. B) View of the propolis envelope when traps were removed at the end of the experiment. In each colony, the bees deposited propolis within most of the gaps of each propolis trap (brown lines on the box are the deposited propolis). In a tree cavity, the propolis envelope is contiguous, but bees do not tend to deposit propolis on planed wooden walls in beekeeping equipment, unless lumber is left unfinished.

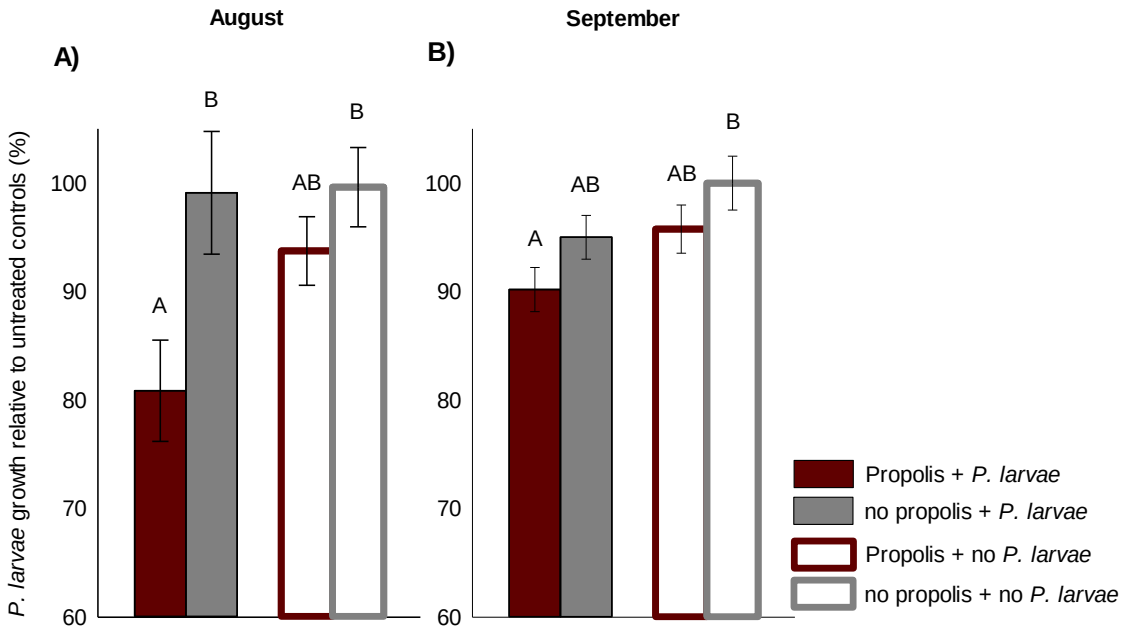

Figure 3.2. Larval food inhibitory activity to *P. larvae* growth. Antimicrobial activity of larval food (average ± SEM) for samples collected in A) August, and B) September were measured as a percent optical density (OD_{600}) relative to untreated controls (N= 32 replicate wells per colony and five colonies per treatment). A low *P. larvae* growth (y axis) indicates higher inhibition activity of larval food. Significant difference between groups, determined by ANOVA with colonies as random variables ($\alpha = 0.05$). Treatment groups not connected by the same letter (A or B) are significantly different.

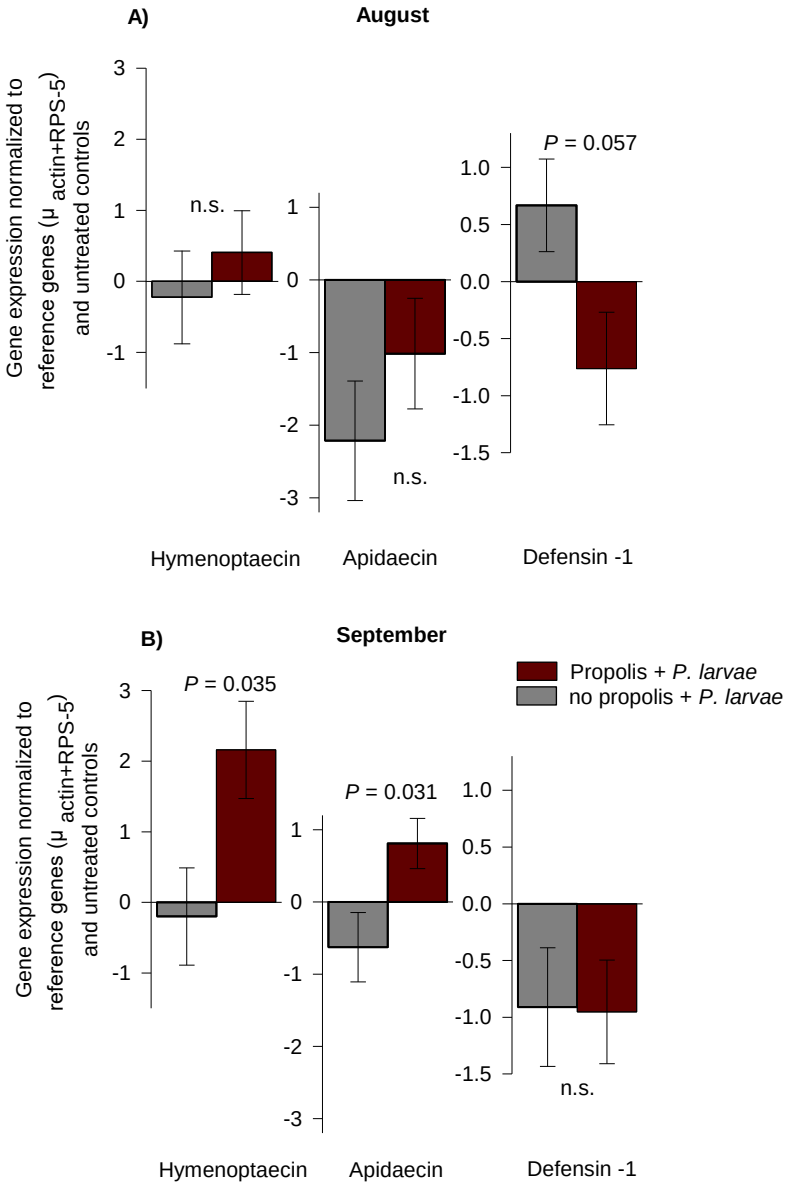

Figure 3.3. Relative expression levels of hymenoptaecin, apidaecin and defensin-1 normalized to reference genes (actin and RPS-5) and untreated controls. Gene transcript average ± SEM (N = 20 biological replicates per colony and 6 colonies per treatment) for A) August, and B) September. Significant difference between groups was determined by two tailed t-test with colonies as random variables ($\alpha = 0.05$). A high value indicates higher gene expression (e.g. in September, challenged colonies with a propolis

envelope had significantly higher expression of both hymenoptaecin and apidaecin compared to challenged colonies without a propolis envelope).

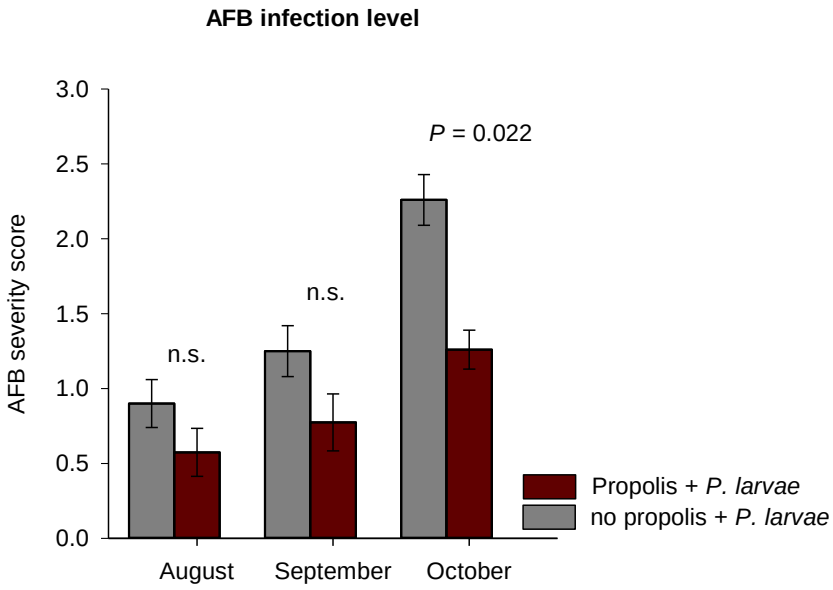

Figure 3.4. American foulbrood infection level. Severity scores (0 = 0 cells containing sign of AFB; 1 = 1-5 cells; 2 = 6-25 cells; and 3 = ≥ 26 cells per comb) average ± SEM (N= 10 colonies) were compared between treatments using two tailed t-test with frames as random variables ($\alpha = 0.05$).

3.8 Tables

Locus	Gene ID	Category	Forward Primer	Reverse Primer	Reference
actin	GB17681	House keeping	TTGTATGCCAACAC TGTCCTTT	TGGCGCGATGATCT TAATTT	Boncristiani et al., 2011
RPS5	GB11132	House keeping	AATTATTTGGTCGC TGGAATTG	TAACGTCCAGCAGA ATGTGGTA	Evans et al., 2006
defensin1	GB19392	Immune	GGATGAATTCGAGC CACTTG	ATGACCTCCAGCTT TACCCA	Designed using MacVector
apidaecin	GI58585226	Immune	TTTTGCCTTAGCAA TTCTTGTTG	GTAGGTCGAGTAGG CGGATCT	Boncristiani et al., 2011
hymenopt	GB17538	Immune	CTCTTCTGTGCCGTT GCATA	GCGTCTCCTGTCAT TCCATT	Evans et al., 2006

Table 3.1. Locus (common) names, gene identification numbers, gene category (development, immune, house keeping), primers sequences and references (when selected from the literature) for genes tested via real time PCR.

Chapter 4.

Resin to Propolis: Biological origins and contribution to honey bee social immunity

Summary

Honey bees collect resins, antimicrobial substances, from plants and incorporate them into the nest architecture. The incorporation of resins from the environment acts as an external immune defense mechanism for honey bees, benefiting colony health by lowering the costly production of some immune-related antimicrobial peptides, and by helping bees fight bacterial and fungal infections. Previous work found that colony infection with the fungal pathogen, *Ascosphaera apis*, elicits bees to increase resin collection (Simone-Finstrom and Spivak, 2012). We tested the robustness of this self-medication response by asking if bees also self-medicate with resin in response to a bacterial infection, American foulbrood (AFB), caused by *Paenibacillus larvae*. We also tested the hypothesis that bees may shift their selection of resin sources at the colony-level after challenge with *P. larvae*, and, if so, how the antimicrobial activity might differ between the pre- and post-challenge resin plant sources. Our results suggested that colonies did not significantly increase resin collection after challenge with *P. larvae*, but they did increase resin foraging after challenge with *A. apis*. We found that after challenge with the fungal pathogen, colonies increased resin collection from the most abundant resin producing tree located in our region, *Populus deltoides*, while *P. larvae* challenged colonies did not increase resin collection to any particular botanical source, compared to unchallenged colonies. Thus, after challenge colonies did not appear to change their foraging preference; instead, bees increased resin foraging from sources of resin previously collected by the colony. Resin from *P. deltoides* had the greatest antimicrobial activity against both *P. larvae* and *A. apis*. This study sheds light on the complex way in which colony-level behavioral defenses contribute to diminish pathogen

infection, and on the role of resins as pharmacological agents in the ecology and evolution of plant-animal interactions.

4.1 Introduction

Plant resins have a number of remarkable biological properties that benefit the plants that produce them (Langenheim, 2003). Just as notable are the ecological interactions and mutualisms that have evolved between plant resins and a wide array of organisms that exploit them. A number of vertebrates collect plant-derived compounds and use them pharmacologically as a defense against their own pathogens and parasites (Gompper and Hoylman, 1993; Gwinner et al., 2000; Petit et al., 2002; Wimberger, 1984). Among invertebrates, the most clear insect examples include parasitoid-infected *Grammia incorrupta* caterpillars that ingest non-nutritive alkaloids (Singer et al., 2009; Smilanich et al., 2011), and *Spodoptera littoralis* and *Spodoptera exempta* caterpillars that preferentially consume high protein diets when infected with a virus or bacteria (Cotter et al., 2011; Lee et al., 2006; Povey et al., 2009; Povey et al., 2014).

Recently, there has been growing interest in how social insects exploit the antimicrobial properties of resin for the health of the colony (Brütsch and Chapuisat, 2014; Castella et al., 2008; Simone-Finstrom and Spivak, 2012). Resin collection in honey bees seems to be both constitutive (collected regardless of physiological demand or pathogen level; Simone et al., 2009; see Chapter 2), and inducible (a conditional response to need or infection; Simone-Finstrom and Spivak, 2012). Inducible resin collection may occur as a form of self-medication, defined as the "defense against pathogens and parasites by one species using substances produced by another species" (Clayton and Wolfe, 1993). Honey bee colonies experimentally challenged with *Ascosphaera apis*, the fungal agent of the larval disease chalkbrood, significantly increase resin foraging compared to control colonies (Simone-Finstrom and Spivak, 2012). These results revealed that bees medicate the colony with resin in response to this particular fungal infection. However, little is known about the mechanisms that influence

resin collection and how widespread self-medication behavior is among social insects (Abbott, 2014).

Wood ants (*Formica paralugubris*) collect variable, but often large, amounts of resins and place them near the brood area (Castella et al., 2008; Brutsch and Chapuisat, 2014). The incorporation of resins into the *F. paralugubris* nest results in reduced growth of microorganisms, significantly reducing the amount of bacteria and fungi present in the nest when compared to resin-deprived colonies (Christe et al., 2003). However, when *F. paralugubris* colonies are challenged with the fungal pathogens *Metarhizium anisopliae* or *Beauveria bassiana*, they do not respond by increasing the rate or quantity of resin collection, or by placing resin closer to the brood (Castella et al., 2008; Brutsch and Chapuisat, 2014). Therefore, the use of resin by this species is considered constitutive, rather than inducible, and not a case of self-medication.

To extend our knowledge of how honey bees use resins, we investigated whether bees self-medicate with resin in response to a bacterial infection of bee brood, American foulbrood (AFB), caused by *Paenibacillus larvae*. Numerous studies have demonstrated that propolis (the beekeeping term for resin after it is deposited in the hive) has antimicrobial properties that inhibit the growth of *P. larvae in vitro* (Bastos et al., 2008; Bilikova et al., 2013; Wilson et al., 2015), indicating that resin could be potentially used therapeutically by bees as a "remedy" to treat this bacterial infection.

We also investigated the possibility that bees select resins from particular botanical sources based on antimicrobial activity, potentially colleting resins with more bioactivity after they are infected with pathogens. There is evidence that vertebrates and invertebrates have evolved to change diet selection in response to parasitism (Kyriazakis et al., 1998; Lee et al., 2006; Singer et al., 2009). When infected with a pathogen or parasite, vertebrates often self-medicate by selecting foods containing effective anti-pathogenic (or anti-parasitic) properties, a behavior called pharmacophagy (Hutchings et al., 2003). To our knowledge bees do not consume resins, but instead, collect and deposit resins within the nest to construct a propolis envelope (Simone-Finstrom and Spivak, 2010). Therefore, a change in resin collection for specific botanical sources would be a

case of pharmacophory, the behavior of "carrying" or "moving drugs" (Simone-Finstrom and Spivak, 2012).

The chemical composition of resins varies qualitatively and quantitatively within and among plants (Witham, 1983). A study of the bioactivity of resins from different botanical sources against *P. larvae* growth revealed significant differences among resins from 14 tree species found on the St. Paul campus of the University of Minnesota in their ability to inhibit the growth of this bacterium (Wilson et al., 2013). However, it is not known if bees selectively collect resins with higher antimicrobial properties. At least two insect species are able to recognize and discriminate among plant resins. Stingless bees are able to recognize resin sources on the basis of several volatile compounds (mono- and sesquiterpenes; Leonhardt et al., 2010), and bark beetles are able to find host trees based on volatile monoterpenes in conifer resin (Wood, 1982; Raffa, 2001). As bees are able to discriminate among resins they may display adaptive plasticity in the collection of resins from particular botanical sources.

We hypothesized that resin collection would increase after *P. larvae* or *A.apis* challenge, as it did after challenge with *A. apis* in Simone-Finstrom and Spivak (2012). We also explored if bees alter their selection of resin to sources with greater biological activity after challenge with a bacterial (*P. larvae*) or fungal (*A. apis*) pathogen.

4.2 Materials and Methods

4.2.1 Experimental colonies and resin foraging activity assessment

This experiment was conducted at the University of Minnesota, Saint Paul campus, over the summers of 2012, 2013 and 2014. Each year, new colonies year were established in small four-frame boxes with sister queens and were equalized for adult bee population size and food stores (honey and pollen). Twenty colonies were established in 2012 and 2013, and 30 were established in 2014. Hygienic tests (following Büchler et al., 2013) were performed on all colonies each year to ensure the colonies did not display hygienic

behavior, a behavioral mechanism of resistance to American foulbrood disease (Spivak and Reuter, 2001), and thus that all colonies would be susceptible to challenge with *Paenibacillus larvae*, the causative agent of American foulbrood.

The number of resin and pollen foragers returning to the hive was quantified by closing the colony once or twice a day (weather depending) for 15 minutes between 1100h and 1600h for 12 observation times over two weeks. These methods followed those of Simone-Finstrom and Spivak (2012), except we doubled the number of observation times for each colony. During each observation, returning foragers with resin loads on the hind legs were captured and held in wire mesh cages until the end of the 15-minute observation time when the total number of resin foragers was recorded for each colony. After resin foragers were collected, all bees at the entrance were photographed in high resolution to obtain an accurate counting of pollen foragers later in the lab. Pollen foragers were used as a proxy for total foraging force, as the pollen loads show clearly the bees have been foraging. In all years, resin loads were collected from each individual resin forager at the end of each 15-minute observation. Resin foragers in the wire cages were chilled on ice for 5 minutes to facilitate the removal of the resin from the forager's hind leg using a fine insect pins (BioQuip No. 2). Each resin load was stored in individual glass vials for analysis of botanical origin.

After the 12 observations each year, 10 of the colonies were challenged with a *P. larvae* spore solution and the other 10 served as controls (unchallenged). Sugar solution containing spores of *P. larvae* was prepared by removing 100 AFB infected scales collected from symptomatic colonies, macerating and suspending the crushed scales in sucrose-water to create a 10^7 spores/ml solution. The concentration was verified using a haemocytometer. Each year, 10 colonies were challenged by spraying 5 ml of the sugar solution containing *P. larvae* spores on each of the four combs within the colony (Seeley and Tarpy, 2007). As controls, 10 unchallenged colonies were sprayed with 5 ml of sugar solution on each comb within the colony.

In 2014, the additional 10 colonies were challenged with *Ascosphaera apis* following the pre-challenge observation period by placing a pollen patty containing

chalkbrood spores on top of the frames, under the cover. Pollen patties were prepared following Jensen et al. (2013). Fifteen black chalkbrood mummies were homogenized in 100 ml distilled water and mixed with 150 g of pollen. *A. apis* unchallenged colonies received pollen patties without spores.

Five days after colonies were challenged with *P. larva*e or with *A. apis*, 12 more 15-min observations were conducted on each colony to quantify resin and pollen foragers during the post-challenge period.

4.2.2 Tracking resin foraging with metabolite fingerprinting analysis

Resin loads from each bee in 2012 and 2014 were dissolved in 10% acetonitrile to a concentration of 1.5 mg/ml for analysis with reverse-phase liquid chromatography mass spectrometry (LC-MS). Samples from 2013 were collected and preserved in glass vial with acetonitrile, but not analyzed due to time and budget constraints. Metabolite fingerprints for resin loads collected in 2012 were generated using high resolution MS operated at 17,500 resolution with (-) ESI ionization (UltiMate 3000 HPLC coupled to a Q-Exactive Fourier transform MS, Thermo-Fisher Scientific, Waltham, MA). Fingerprints for resin loads collected in 2013 and 2014 were generated using unit-mass resolution MS with both (+) and (-) ESI ionization (Acquity UPLC coupled to SQD MS, Waters, Milford, MA). The same LC parameters were used for all samples (gradient: 0.1% formic acid in water to acetonitrile, column: Zorbax Eclipse XDB C18 (Agilent Technologies, Santa Clara, CA), 2.1 x 100 mm, 1.8 μm particle size, flow rate: 350 μL/min).

4.2.3 Antimicrobial assays

Two different dilution assays were performed to assess the inhibitory activity of bee-collected resin to the growth of the honey bee bacterial pathogen *P. larvae*, and the fungal pathogen *A. apis* for samples collected in 2012 and 2014, according to Wilson et al. (2015).

4.2.3.1 Bacterial inhibition assay

Four randomly selected resin loads from each botanical source, as identified by metabolite fingerprinting analysis, were tested for inhibitory activity. Resins were diluted in acetonitrile, transferred into 96 well plates, dried under nitrogen gas, and resolubilized in 100 µL of brain/heart infusion broth for 30 min. The final concentrations in wells ranged from 8 mg/l to 175 mg/l. *P. larvae* (from stock strains obtained from the UDSA Agricultural Research Service culture collection; NRRL# B-2605) were cultured overnight in 30 ml of brain/heart infusion (BHI) broth, diluted 1:100 in fresh BHI, and transferred into the well plates. Plates were incubated in a microplate incubator shaker at 37 C and 400 rpm for six hours. Bacterial growth inhibition was evaluated by measuring turbidity (optical density at time 0 h subtracted from time 6 h, OD600) of treated cultures relative to untreated controls using a microplate spectrophotometer.

4.2.3.2 Fungal inhibition assay

The same randomly selected resin loads tested in the bacterial growth inhibition assay were also tested against *A. apis*. Resins were diluted in acetonitrile, transferred into a different set of 96 well plates, dried under nitrogen gas, and resolubilized in 180 µL of liquid MY-20 media for 30 min. Final concentrations in well plates ranged from 0.25 mg/l to 175 mg/l. *A. apis* reference strains were obtained from the ARS Entopathogenic Fungal Culture Collection (http://www.ars.usda.gov/is/np/systematics/fungibact.htm) [USDA #7405 (ATCC MYA-4450, mating type +) and USDA #7406 (ATCC MYA-4451, mating type -)]. Fungi were grown and mated on MY-20 media with spores isolated according to standard methods (Jensen et al., 2013). Each microplate well was inoculated with 1.98 x 10^6 spores in 20 uL of sterile water. Vegetative growth of *A. apis* was measured relative to untreated controls after 72 h of incubation at 31 C and 400 rpm.

4.2.4 Statistical analysis

The change in resin and pollen foragers between the pre- and post-challenge periods was calculated for each colony by subtracting the total number of foragers before challenge from the total number of foragers after challenge. In 2012 and 2013, the difference between treatment groups (challenged and not challenged) was compared using two tailed t-tests with treatment as a fixed effect using R version 2.15. In 2014, with the addition of a third treatment group, *A. apis* challenge, the difference among groups was compared using ANOVA followed by Tukey-HSD test. Data for resin collection by colonies challenged with *P. larvae* (and separately for resin collection by unchallenged control colonies) were combined across years and analyzed using ANOVA with year and treatment as fixed effects. The difference in number of resin foragers for each botanical source of resin was evaluated as described for total resin foraging.

The Genedata Expressionist for Mass Spectrometry software package (http://www.genedata.com/products/expressionist/mass-spectrometry.html) was used to extract mass/retention time pairs from the raw LC-MS to create a peak matrix for statistical analysis (Wilson et al., 2015). Principal components analysis (PCA) was used to group samples based on the covariance of extracted mass/retention time pairs among resin samples. Resin loads that clustered close together relative to others were deemed to have the same botanical source. Resin load groups were verified by randomly selecting five samples from each PCA group, visually inspecting fingerprints for similarity, and then checked for the presence of plant-specific markers (Wilson et al., 2015).

IC_{50} values for resin loads was calculated by fitting a four-parameter logistic equation to the sigmoidal inhibition curves using Sigmaplot 12.5 (Systat Software Inc., Chicago IL). The IC_{50} values were compared using pair-wise comparison ($\alpha = 0.05$) to determine statistical differences in resin bioactivity among botanical sources. Values were compared pair-wise using confidence intervals, calculated as: $\mathrm{CI} = \mathrm{Z} \pm [1.96 \times (\sqrt{x^2 + y^2})]$. Where x is the standard error of $IC_{50}(1)$, y is the standard error of $IC_{50}(2)$, and Z is the difference between $IC_{50}(1)$ and $IC_{50}(2)$. If the confidence interval did not include 0, the difference between the two IC_{50} values was considered statistically significant.

4.3 Results

4.3.1 Resin and pollen foraging activity

Resin foraging by bees in colonies challenged with *P. larvae* did not significantly increase after colony inoculation, compared to control colonies, in any of the three years (Figure 4.1). Combining the three years of data, the numerical increase in resin foraging after *P. larvae* challenged was not significantly significant increase (Figure 4.1d). In contrast, in 2014, bees in colonies challenged with *A. apis* had a significant increase in the number of resin foragers after colony inoculation compared to controls ($F_{2,27} = 3.63$, $P = 0.04$; Figure 4.1c). The average number (± standard deviation) of resin foragers recorded before and after colony inoculation (12, 15-minute observation times for each period) is reported in Table 4.1.

In each of the three years, there was no significant difference in the number of pollen foragers before and after challenge, indicating that increase in resin foragers after challenge was not due to an increase in overall foraging effort (Figure 4.2). The number of pollen foragers was 5-6 times greater than resin foragers in 2012, 3 times greater in 2013 and 6-9 times greater in 2014 (Table 4.2).

4.3.2 Resin foraging activity by botanical source

In 2012 and 2014, bees collected resin from *P. deltoides* (Eastern cottonwood), *P. hybrid* (Poplar hybrid), and three unknown sources (unknown 1, 2 and 3). All three unknown source metabolic fingerprints did not match the patterns of any previously sampled tree species from around the St. Paul campus of the University of Minnesota (a total of 14 species reported in Wilson et al., 2013), suggesting that bees collected resins from plants in the diverse urban landscape surrounding campus.

In 2012, *P. larvae* challenged colonies did not collect more resin from any of the four botanical sources after challenge, compared to control colonies (Figure 4.3a). Bees from the *P. larvae* challenged and control colonies did not forage for *P. hybrid* resin after colony inoculation in 2012 (although they did collect it before challenge); therefore, resin foraging activity for *P. hybrid* decreased for both treatment groups (Figure 4.3a).

In 2014, resin foraging for botanical source unknown 1 increased in *P. larvae* challenged colonies compared to *A. apis* challenged colonies, but not control colonies ($F_{2,27} = 4.54$, $P = 0.02$; Figure 4.4a). Statistical differences were found in number of resin foragers to Unknown 1 botanical source in 2014 (Figure 4.4a), but there were so few resin foragers to this plant species (ranging from 1-2 resin loads per colony per observation period, see Figure 4.4a), it is not clear if the differences are of biological significance.

A. apis challenged colonies collected significantly more resin from *P. deltoides* trees after challenge compared to controls, but *P. larvae* challenged colonies did not collect more resin from this species ($F_{2,27} = 4.38$, $P = 0.02$; Figure 4.4a). No colonies from the three treatment groups increased resin foraging for *P. hybrid* or Unknown 3 (Figure 4.4a).

4.3.3 Resin antibacterial and antifungal activity

We explored whether resin foragers change the type of resin they collect after pathogen challenge, possibly collecting resins of higher antimicrobial activity. To do this, we measured the *in vitro* activity of all collected resin species against *P. larvae*. Only resins collected in 2014 were tested against *A. apis*, as this was the only year that included a treatment group of colonies challenged with *A. apis* (Figures 4.3b and 4.4b).

In 2012, resins from the four botanical sources varied in their ability to inhibit *P. larvae* (Figure 4.3b). Based on pairwise comparisons of confidence intervals, resin from *P. deltoides* and Unknown 2 had significantly higher inhibitory activity (lower IC_{50}) against *P. larvae* growth compared to resins from *P. hybrid* and Unknown 1 (Table 4.3).

In 2014, resin from *P. deltoides* and Unknown 3 had similar and significantly higher *in vitro* activity against *P. larvae* compared to resin from *P. hybrid* and Unknown 1 (Table 4.3).

Resin from the same botanical sources varied in their ability to inhibit *P. larvae* and *A. apis* (Figure 4.4b). Resins from *P. deltoides* and *P. hybrid* had the highest inhibitory activity against *A. apis*, and resins from Unknown 3 were the least inhibitory (Table 4.3). Resins from Unknown 1 showed intermediate inhibitory activity, although significantly different than the most and least inhibitory resin samples (Table 4.3).

4.4 Discussion

Our results do not support the hypothesis that bees self-medicate in response to a bacterial infection from *P. larvae*. Over the three years of this study, we observed a numerical increase in resin foragers after challenge with the bacterial pathogen *P. larvae*, the causative agent of American foulbrood disease. But in no year alone, or when the three years combined, was there a statistically significant increase in resin foraging in response to infection with this bacterial pathogen. Our findings do support previous results that bees self-medicate with resin in response to fungal infection from *A. apis* (Simone-Finstrom and Spivak, 2012). After challenge with this fungal pathogen, colonies increased resin collection from the most common resin producing tree located in our region, *Populus deltoides* (cottonwood trees), while *P. larvae* challenged colonies did not change resin collection to any particular botanical source. Of the three different plant sources of resin collected by colonies challenged with *A. apis*, the resin from *P. deltoides* had the greatest antimicrobial activity against that fungal pathogen.

It is unclear if there are biological reasons why bees do not collect more resin in response to bacterial infection compared to fungal infection. It seems unlikely that bees have evolved to collect resin in response to volatile compounds produced by fungi or fungal-infected brood but not in response to bacteria or bacterial-infected brood. Honey

bees naturally nest in tree cavities where they prepare the new nest site by removing the soft, rotten wood from the nest walls and deposit propolis in the cracks and top surface to make it solid and smooth (Seeley and Morse, 1976). Early microorganisms invaders of tree cavities, including bacteria, weaken tree defenses and allow further invasion by fungi, causing wood decay (Carey, 1983; Shigo, 1985; Jackson and Jackson, 2004). The intimate association of bacterial and fungal decay and honey bees nesting in tree cavities suggests that bees have evolved to detect volatiles produced by both bacteria and fungi, and in response, deposit propolis on the nest walls. Bees are able to detect the volatiles produced by brood infected with both the bacterial pathogen *P. larvae*, and the fungal pathogen *A. apis*, and perform hygienic behavior towards both types of infected brood, (Rothenbuhler, 1964; Spivak and Gilliam, 1998; Spivak and Reuter, 2001; Swanson et al., 2009). Thus, it seems that if bees are able to self-medicate to a fungal infection they should also be able to self-medicate to a bacterial infection, but our data did not support this hypothesis.

It could be the statistical power of our test was limited by the low number of resin foragers in each colony. Resin foraging is particularly rare, compared to nectar and pollen foraging. It is estimated that the number of resin foragers is less than 1% of the total number of foragers in the hive and that this foraging preference may be influenced by the bees' genetics (Butler, 1949; Page and Fondrk, 1995). *A. apis* challenged colonies significantly increased resin foraging after challenge compared to control colonies, but not to *P. larvae* treatment colonies. If the presence of *P. larvae* in the colony does not result in a high increase in resin foraging, a greater number of colonies might be necessary to increase the statistical power of our analyses, given the high variance in the total number of resin foragers among colonies. Based on the data we collected, we are not able to say that bees self-medicate by collecting more resin after *P. larvae* challenge.

The increase in resin collection we observed in colonies challenged with *A. apis* was not due to an increase in general foraging activity, as the number of pollen foragers did not change after colony inoculation. In all three years, the number of resin foragers was considerably lower compared to pollen foragers. Pollen foragers were counted after 3

minutes within each 15-minute observation time, and the number of bees carrying pollen was 3 to 9 times higher in 3 minutes compared to the number of resin foragers over the 15-minute observation time.

We investigated if bees collect resin from different tree species after pathogen challenge, potentially selecting for resins with greater bioactivity. We found that *A. apis* challenged colonies increased resin collection for the most common resin producing tree located in our region, *Populus deltoides* (Eastern cottonwood trees; Wilson et al., 2013). Resin from *P. deltoides* was the most predominant type of resin collected by all colonies, and compared to the other botanical sources bees were collecting, resin from *P. deltoides* had the highest antimicrobial activity against both *P. larvae* and *A. apis*. Thus, after challenge colonies do not appear to change their foraging preference; instead, it appears that bees simply increase resin foraging for resin sources previously collected by the colony. In fact, other trees in the area, such as white spruce (*Picea glauca*), secrete resin with even higher antimicrobial activity against *P. larvae* compared to Eastern cottonwood (Wilson et al., 2013), but we have never collected resin from bees' legs that originated from white spruce, so it is unclear if they even visit this tree for resin. The decision making process for the recruitment of specific resin sources after chalkbrood and American foulbrood infection, and whether the decisions are driven by plant resin source abundance or resin bioactivity, requires further investigation.

The prevalence of foragers with *P. deltoides* resin loads was observed in all colonies, before and after challenge (from 2 to 42 bees per colony over each observation period). Resin from the other plant sources was not collected in great quantities (from 0-4 bees per colony for Unknown 2 and 0-2 bees for Unknown 1 and 3 over each observation period). The metabolic fingerprints of resins from the three unknown sources did not match the patterns of any previously sampled tree species from around the St Paul campus of the University of Minnesota (a total of 14 species reported in Wilson et al., 2013). The St. Paul campus of the University of Minnesota is surrounded by urban gardens and landscaping, with diverse species of plants that the bees may be finding and using as resin sources. Without locating the plant source, we cannot confirm the identity

of the resin using our techniques (liquid chromatography followed by mass spectrometry; LC-MS). Resin foraging is difficult to observe, because bees often collect resin high in the canopy of trees, which makes it difficult to identify the botanical source of resin directly. Other techniques, such as TLC and TLC-MS, have been used to identify the botanical sources of resin or propolis (e.g. Bertrams et al., 2013). Results from these other methods can inform us of potential botanical sources of our unknowns, but our LC-MS data are not directly comparable to identifications produced using other methods, so exact identifications are not possible.

The behavioral mechanisms that initiate resin collection, and how bees recruit each other to resin sources, are not well known. The number of foragers allocated to a source is likely a direct result of recruitment to that botanical source. Individual bees may detect the need for resin, and then after collecting it use communication signals (e.g., waggle dances, trembling) inside the nest to recruit nest mates to forage for more resin from a specific tree species (Nakamura and Seeley, 2006), as they do to recruit nest mates to food resources. Honey bee foragers are able to assess the quality of the nectar source by taking into account the distance of the flower patch to the hive, the sugar concentration of the nectar and the nectar abundance (Seeley et al., 1991). However, it is unknown which and how many factors influence recruitment to resin sources, particularly since bees do not consume the resins. It is possible that bees assess the quality of the resin not just based on its antimicrobial properties, but also the abundance of that resin and the amount of energy allocated to collect the resin (distance to the hive and time to remove the resin from the plant).

More studies are needed to explore how pathogen infection induces resin collection and recruitment. Our study provides insight into the complex way in which colony-level behavioral defenses contribute to reduce pathogen infection, and on the role of resins as pharmacological agents in the ecology and evolution of plant-animal interactions.

4.5 Conclusion

The adaptive plasticity of resin collection for one pathogen, and not the other, may be a result of the evolution of host-pathogen interactions. The coevolution between bees and their pathogens have resulted in the development of a set of behavioral defense mechanisms by which bees limit pathogen infection using a less costly mechanism than the activation of the immune system. One of many of these behaviors is resin collection, which we suggest to occur to prevent (prophylaxis; e.g. Simone et al., 2009; Chapter 2) or to treat an infection (therapeutic medication; e.g. Simone-Finstrom and Spivak 2012; Chapter 4). Although *A. apis* challenged colonies significantly increase resin collection for one botanical source (*P. deltoides*), we were unable to determine whether the selection was due to the quality of the resin (highest bioactivity against *A. apis* growth) or the abundance of this tree within bees flight range, as *P. deltoides* was the most common resin-producing tree in our research apiary location. Our results emphasize the importance of resin to bees and show that plants are not only a source of food but can also be "pharmacies."

4.6 Acknowledgments

We would like to thank Gary Reuter and Christine Kulhanek (University of Minnesota) for assistance with honey bees' colony management, and Dr. Michael Simone-Finstrom (USDA) for providing advice on some of the experimental procedure. We also acknowledge the support of all the members of the Bee Lab at University of Minnesota.

4.7 Figures

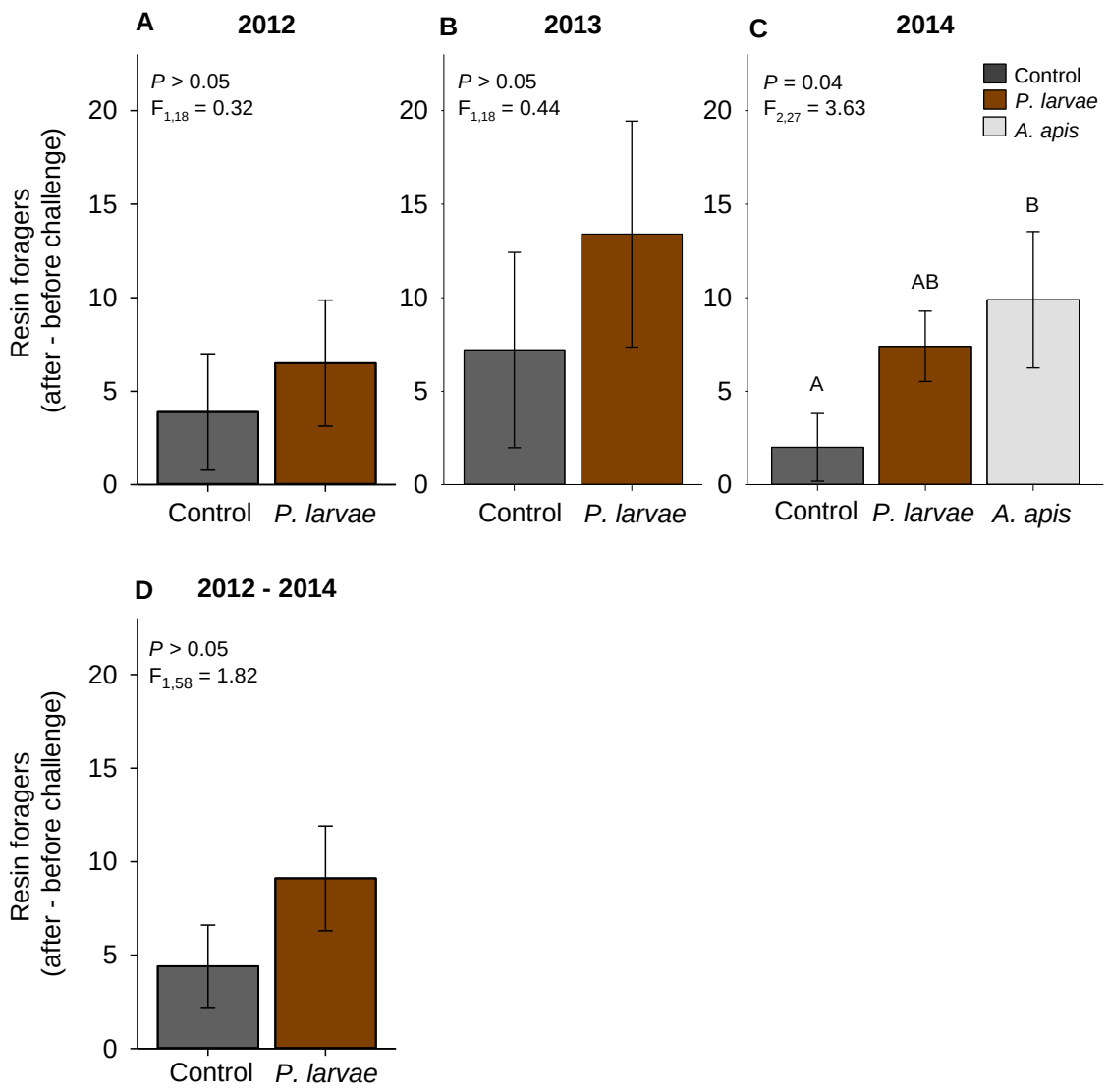

Figure 4.1. Resin foraging activity ± SE (N= 10 colonies per treatment) measured as the difference of resin foragers between after and before colony inoculation for A) 2012, B) 2013, C) 2014 and D) Combined data for *P. larvae* and unchallenged treatments over the three years of this study. Significant differences between treatment groups was determined by two tailed t-test in 2012 and 2013 and by ANOVA followed by Tukey-HSD test in 2014. Treatment groups designated with different letters (A, B) are significantly different.

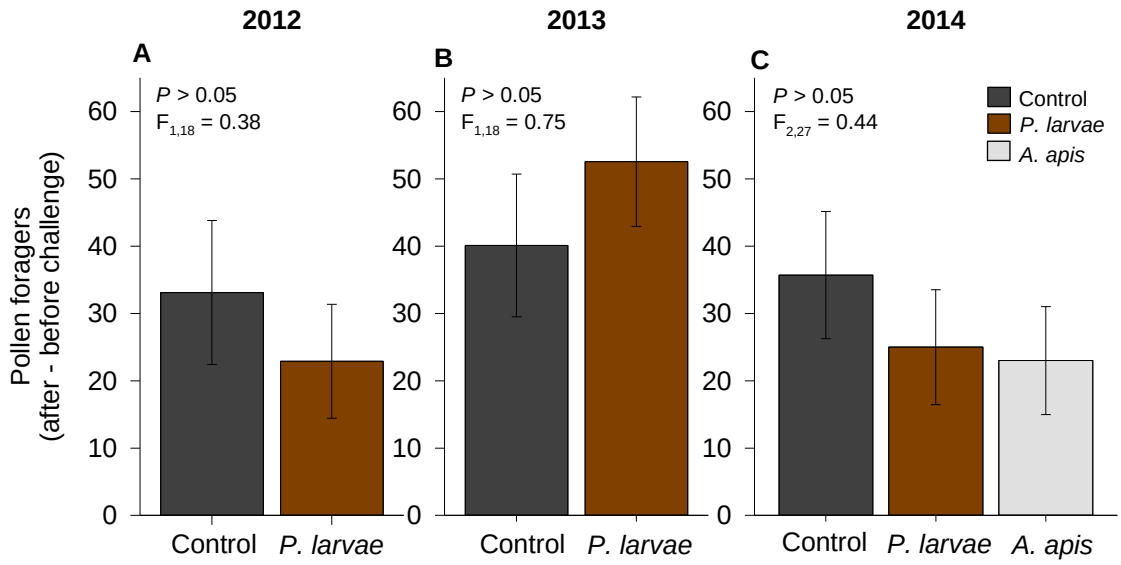

Figure 4.2. Pollen foraging activity ± SE (N= 10 colonies per treatment) measured as the difference of pollen foragers between after and before colony inoculation for A) 2012, B) 2013, and C) 2014. Significant difference between treatment groups was determined by two tailed t-test.

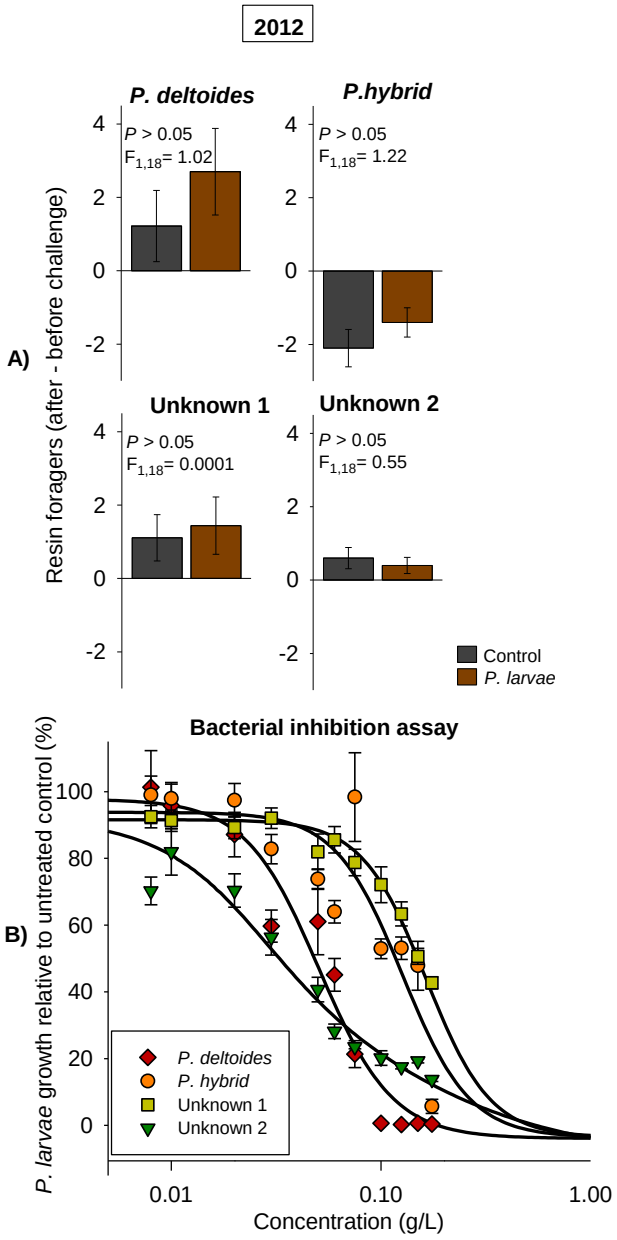

Figure 4.3. Resin foraging activity by botanical source and resin antibacterial activity for samples collected in 2012. A) Resin foraging activity ± SE (N= 10 colonies per treatment) for each botanical source measured as the difference of resin foragers between after and before colony inoculation, and followed by t-tests between groups. B) Resin

bioactivity against *P. larvae* measured as a percent optical density (OD_{600}) relative to untreated controls (N= 4 replicate wells per botanical source).

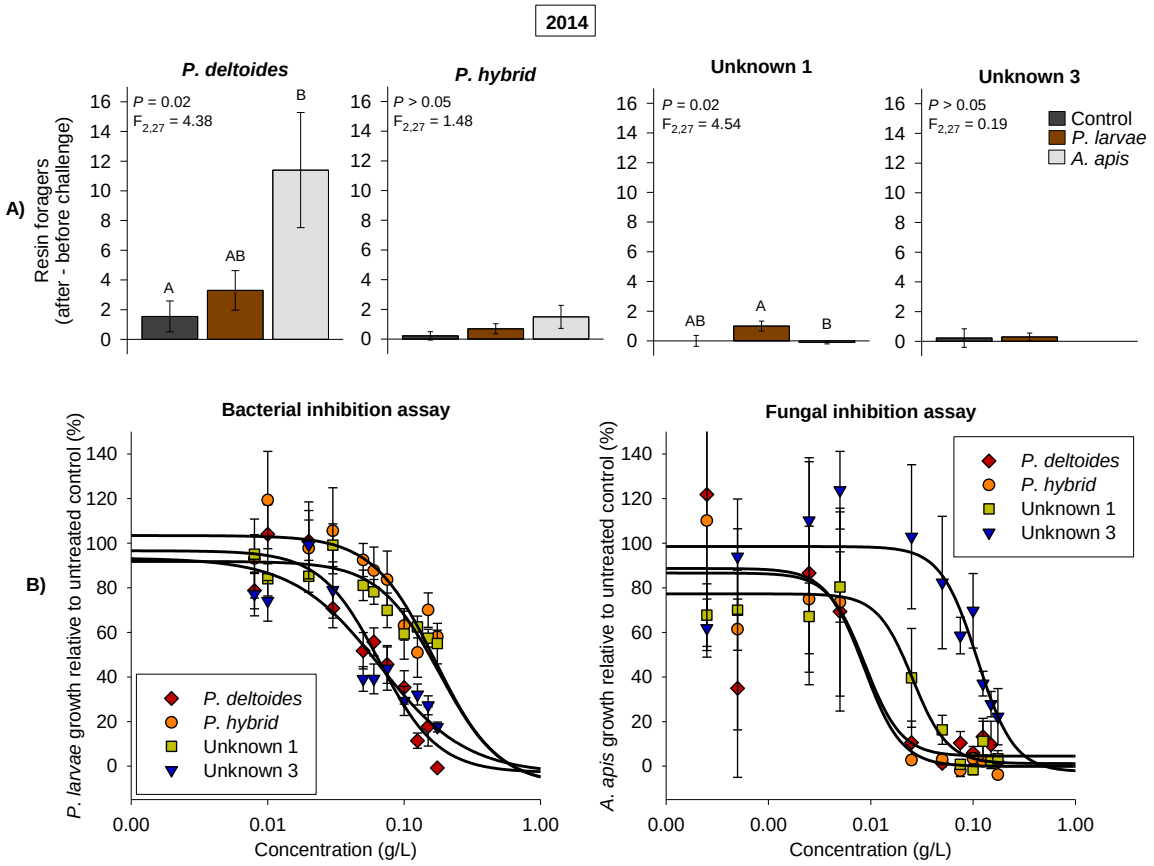

Figure 4.4. Resin foraging activity by botanical source and resin antibacterial activity for samples collected in 2014. A) Resin foraging activity ± SEM (N= 10 colonies per treatment) for each botanical source measured as the difference of resin foragers between after and before colony inoculation, and followed by ANOVA among groups and Tukey-HSD test. Treatment groups not connected by the same letter (A, B) are significantly different. B) Resin bioactivity against *P. larvae* and *A. apis* measured as a percent optical density (OD_{600}) relative to untreated controls (N= 4 replicate wells per botanical source).

4.8 Tables

	Unchallenged		***Paenibacillus larvae***		***Ascosphaera apis***	
Year	Before	After	Before	After	Before	After
2012	12.1 ± 5.8	16.0 ± 7.9	9.4 ± 3.7	15.9 ± 9.5	-	-
2013	26.8 ± 19.3	34.0 ± 21.7	15.5 ± 9.6	28.9 ± 17.7	-	-
2014	7.2 ± 4.3	9.0 ± 2.3	4.9 ± 2.9	12.3 ± 7.9	7.4 ± 2.7	18.1 ± 11.2

Table 4.1. Average number of resin foragers (mean ± S.D, and data range) in unchallenged (control; N= 10 colonies/year), *P. larvae* (N= 10 colonies/year) and *A. apis* colonies (N= 10 colonies in 2014 only). Each before and after period consisted of a total of 12, 15-minute observations.

	Unchallenged		***Paenibacillus larvae***		***Ascosphaera apis***	
Year	Before	After	Before	After	Before	After
2012	70.0 ± 55.8	103.1 ± 36.3	56.8 ± 17.0	79.7 ± 31.5	-	-
2013	52.2 ± 14.2	92.3 ± 34.5	41.1 ± 16.5	92.1 ± 27.1	-	-
2014	57.6 ± 14.1	91.7 ± 11.2	60.9 ± 14.0	86.11 ± 12.4	50.8 ± 13.4	74.0 ± 19.1

Table 4.2. Average number of pollen foragers (mean ± S.D, and data range) in unchallenged (control; N= 10 colonies/year), *P. larvae* (N= 10 colonies/year) and *A. apis* colonies (N= 10 colonies in 2014 only). Each before and after period consisted of a total of 12, 15-minute observations.

Resin botanical source	*P. larvae* IC_{50} value (g/l)		*A. apis* IC_{50} value (g/l)
	2012	2014	2014
Populus deltoides	0.07 ± 0.0068 (a)	0.05 ± 0.009 (a)	0.0043 ± 0.002 (a)
Populus hybrid	0.11 ± 0.018 (b)	0.126 ± 0.041 (b)	0.005 ± 0.001 (a)
Unknown 1	0.15 ± 0.014 (b)	0.182 ± 0.045 (b)	0.022 ± 0.004 (b)
Unknown 2	0.06 ± 0.036 (a)	-	-
Unknown 3	-	0.06 ± 0.005 (a)	0.12 ± 0.029 (c)

Table 4.3. IC_{50} values of bee-collected resin inhibiting *P. larvae* (2012 and 2014) and *A. apis* (2014 only) calculated from dose–response curves. Values were calculated by fitting data points with a four-parameter curve in SigmaPlot 10.5. Statistical differences among species based on non-overlapping 95% confidence intervals for the differences between IC_{50} values are indicated by different letters following each IC_{50} value (within each column).

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